Crosstalk of the HGF/c-MET and TGF-β Pathways in Glioblastoma

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IV. Abbreviations

ACVR	activin receptor
ALK	activin receptor-like kinase
AMH	anti-mullerian hormone
AP	alkaline phosphatase
ATRX	alpha-thalassemia/mental retardation syndrome X-linked
BBB	blood brain barrier
BCA	bicinchoninic acid assay
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
CAR	chimeric antibody receptor
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CDKN	cyclin-dependent kinase inhibitor
CNS	central nervous system
Co-SMAD	common mediator SMAD
CRK	v-crk sarcoma virus CT10 oncogene homolog
CRKL	CRK-like
CSC	cancer stem cells
CTL	cytotoxic T-lymphocyte
CTLA	cytotoxic T-lymphocyte-associated antigen
DG	dentate gyrus
DMSO	dimethylsulfoxide
E2F	E2 factor
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor

G-CIMP	CpG-island methylator phenotype
GAB	GRB2-associated binding protein
GDF	growth differentiation factors
GIC	glioma-initiating cells
GRB	growth factor receptor-bound protein
H3-K27M	histone 3 lysine 27-to-methionine
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HL	hairpin loop
HLA-DR	human leukocyte antigen D related
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradish peroxidase
ID	inhibitor of DNA binding protein
IDH	isocitrate dehydrogenase
IGFBP	insulin-like growth factor-binding protein
ICC	immunocytochemistry
IL	interleukin
IHC	immunohistochemistry
IPT	Immunoglobulin-plexin-transcription
I-SMAD	inhibitory SMAD
IκBα	inhibitor of NF-κB
JNK	JUN amino-terminal kinase
KLF	kruppel-like factor
L1CAM	L1 cell adhesion molecule
LAP	latency-associated peptide
LIF	leukemia inhibitory factor
LLC	large latent TGF-β complex
LTBP	latent TGF-β binding protein
LV	lateral ventricle
MAPK	mitogen-activated protein kinase
MDM	murine double minute
MeA	methyladenine
MeG	methylguanine

MGMT	O^6 -methylguanin-DNA methyltransferase
MH	MAD homology
miR	micro RNA
MMP	matrix metalloproteinase
MMR	mismatch repair
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
NBM	neurobasal media
NSC	neural stem cells
NF-κB	nuclear factor kappa B
NK	natural killer
NKG2D	NK group 2 member D
OCT	octamer-binding transcription factor
p14ARF	alternative reading frame protein 14
PAI	plasminogen activator inhibitor
PARP	poly-adenosine diphosphate ribose polymerase
PBS	phosphate-buffered saline
PD	programmed death
PDGFR	platelet-derived growth factor
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PSI	plexin–semaphorin–integrin
PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatases
RB	retinoblastoma-associated protein
RIPA	radio-immunoprecipitation assay
RISC	recurrence initiating stem-like cancer
R-SMAD	receptor-activated SMAD
RTK	receptor tyrosine kinase
RT-PCR	real-time polymerase chain reaction

SBE	SMAD binding element
SEMA	semaphorin
SGZ	subgranular zone
SHC	src homology 2 domain-containing
SHH	sonic hedgehog
SHP2	src homology 2 domain-containing phosphatase 2
SLC	small latent TGF-β complex
SMURF	SMAD specific E3 ubiquitin protein ligase
SOX	sex determining region Y-box
SP	signal peptide
SPH	serine protease homology
SRC	v-src sarcoma viral oncogene homolog
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
TAK	TGFβ-activated kinase
TCR	T-cell receptor
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor-β
TGF-βR	transforming growth factor-β receptor
THBS	thrombospondin
TIMP	tissue inhibitor of metalloproteinase
TMZ	temozolomide
TNF	tumor necrosis factor
TP53	tumor protein p53
TRAF	tumor receptor-associated factor
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
WHO	world health organization

1. Summary

The prognosis of glioblastoma, the most common type of intrinsic brain tumor, remains poor even with the current standard of care of the combination of surgery, radiotherapy, and chemotherapy. Multiple target inhibition has gained considerable interest in combating drug resistance in glioblastoma. However, understanding the molecular mechanisms of crosstalk between signaling pathways and predicting responses of cancer cells to targeted interventions has remained challenging.

Among the multiple signaling pathways associated with glioblastoma, the hepatocyte growth factor (HGF)/c-MET and transforming growth factor (TGF)-β pathways have gained particular attention because of their putative roles in glioblastoma stem cell function, in the development of invasiveness and resistance to radio- and chemo-therapy as well as targeted therapies. Despite the significant role attributed to HGF/c-MET and TGF-β signaling in glioblastoma pathogenesis, their functional interactions have not been well characterized. In this thesis, using genetic and pharmacological approaches to stimulate or antagonize the TGF-β-pathway in human glioma-initiating cells (GIC), we observed that TGF-β exerts an inhibitory effect on HGF/c-MET signaling, which is mediated by mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK). Moreover, TGF-β control of HGF/c-MET is also regulated by phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/AKT) pathway. A comparison of c-MET-driven and c-MET independent GIC models revealed that TGF-β inhibits stemness in GIC at least in part via its negative regulation of c-MET activity, suggesting that the balance between these two oncogenic pathways may control stem cell maintenance. Importantly, immunohistochemical analyses of TGF-β and p-c-MET in human glioblastoma specimens support a concept of negative regulation between these pathways.

These novel insights into the crosstalk of two major pathogenic pathways in glioblastoma may explain some of the disappointing results when targeting either pathway alone in human glioblastoma patients and inform on potential future designs on targeted pharmacological or genetic intervention.

2. Zusammenfassung

Die Prognose des Glioblastoms, des häufigsten Typs eines intrinsischen Hirntumors, bleibt auch bei dem derzeitigen Therapiestandard der Kombination von Chirurgie, Strahlentherapie und Chemotherapie schlecht. Zielgerichtete Therapien haben grosses Interesse auch bei der Űberwindung der Therapieresistenz beim Glioblastom hervorgerufen. Das Verständnis der molekularen Mechanismen der Interaktionen zwischen den verschiedenen Signalwegen und die Vorhersage der Reaktionen von Tumorzellen auf zielgerichtete Interventionen bleibt aber unvollständig.

Unter den multiplen Signalwegen, die bei Glioblastomen verändert sind, haben die Signalwege des Hepatozyten-Wachstumsfaktors (HGF) und seines Rezeptors c-MET und des Transformierenden Wachstumsfaktors (TGF)-β aufgrund ihrer vermeintlichen Rollen für Glioblastom-Stammzellen und die Entwicklung von Invasivität und Resistenz gegenüber Strahlen- und Chemotherapie besonderes Interesse hervorgerufen. Trotz der signifikanten Rolle, die diesen Wachstumsfaktor-Signalwegen bei der Glioblastom-Pathogenese zugeschrieben wird, sind ihre funktionellen Interaktionen nicht gut charakterisiert.

In dieser Arbeit wurde mit Hilfe genetischer und pharmakologischer Ansätze zur Stimulierung oder Antagonisierung des TGF-β-Signalweges in humanen Gliom-initiierenden Zellen (GIC) nachgewiesen, dass TGF-β eine hemmende Wirkung auf die HGF/c-MET-Signaltransduktion ausübt, welche durch mitogen-aktivierte Proteinkinase (MAPK/ERK) vermittelt wird. Ebenfalls wird diese Regulierung des HGF/c-MET-Signalwegs durch TGF-β durch Phosphatidylinositol-3-Kinase (PI3K)/protein kinase B (PKB/AKT)-Signalwege kontrolliert.

Desweiteren zeigte ein Vergleich von c-MET-abhängigen und c-MET-unabhängigen GIC-Modellen, dass TGF-β die Stammzelleigenschaften in GIC zumindest teilweise über die negative Regulierung der c-MET-Aktivität hemmt. Dies deutet darauf hin, dass der Stammzell-Pool durch das Gleichgewicht zwischen diesen beiden onkogenen Signalwegen kontrolliert wird. Die immunhistochemischen Analysen von TGF-β und phosphoryliertem c-MET in humanen Glioblastomschnitten unterstützen die Hypothese der negativen Regulation zwischen diesen Signalwegen.

Diese neue Betrachtung der Interaktion zwischen zwei wichtigen pathogenen Signalwegen im Glioblastom können einige enttäuschende Ergebnisse erklären,

wenn entweder TGF-β oder HGF/c-MET allein beim Glioblastom gehemmt wurde, und zeigt auf, wie zukünftige gezielte pharmakologische oder genetische Interventionen erfolgreicher verlaufen könnten.

3. Introduction

3.1 Glioblastoma

3.1.1 The 2016 World Health Organization classification of the gliomas

Gliomas represent 80% of all malignant primary brain tumors and are responsible for the majority of brain tumor-related deaths (Weller *et al.*, 2015). The 2016 WHO classification of tumors of the central nervous system (CNS) categorizes gliomas into distinct entities according to integrated histological and molecular criteria, which provides a more accurate and reproducible diagnostic system (Reifenberger *et al.*, 2016). Prior to the 2016 WHO classification, gliomas were solely grouped into four histopathological and prognostic grades (WHO grade I-IV). WHO grade I defines non-infiltrative, slow-growing lesions with favourable prognosis. WHO grade II indicates slow-growing but infiltrative tumors that usually recur and tend to progress to higher grades. WHO grade III lesions are characterized by nuclear atypia and high mitotic activity. WHO grade IV designation indicates the most malignant and incurable tumors (Louis *et al.*, 2007). Grade II-IV tumors exhibit diffuse invasion into the surrounding tissue without demarcated margins. The main advance of the 2016 WHO classification is the incorporation of isocitrate dehydrogenase 1 or 2 (IDH)-mutations, nuclear expression of the alpha thalassemia/mental retardation syndrome X-linked (ATRX), short arm of chromosome 1 (1p)/long arm of chromosome 19 (19q) co-deletion and histone 3 lysine 27 to-methionine (H3-K27M)-mutation as diagnostic biomarkers to define glioma subtypes. The revised classification of diffuse gliomas includes: IDH-mutant and 1p/19q-codeleted oligodendroglioma of WHO grade II-III, IDH-mutant diffuse astrocytoma of WHO grade II-III, IDH-wild-type glioblastoma of WHO grade IV, IDH-mutant glioblastoma of WHO grade IV, and H3-K27M-mutant diffuse midline glioma of WHO grade IV (Reifenberger *et al.*, 2016) (Fig. 1).

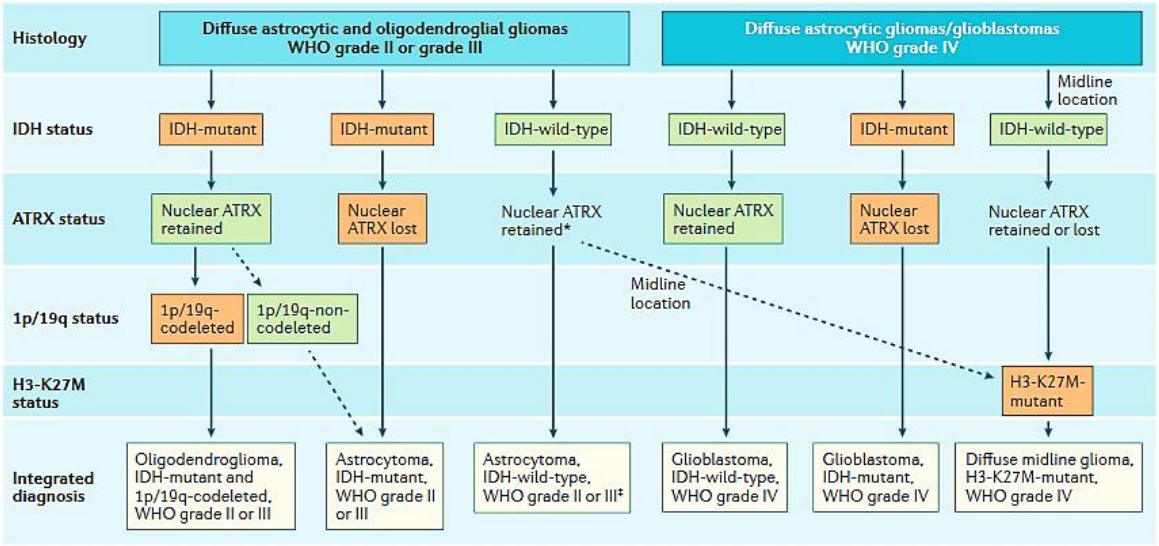


Figure 1. Diagnostic approach for integrated histological and molecular classification of diffuse gliomas according to the 2016 WHO classification of tumors of the CNS. The integrated analysis of IDH-mutation status together with histologic feature define glioma subtypes. ‡ IDH-wild-type astrocytomas of WHO grade II or III are considered provisional entities. The presence of 1p/19q codeletion in IDH-mutant patients maintaining nuclear ATRX expression constitute additional parameters for tumor classification. Further, H3-K27M mutations can be assessed in IDH-wild-type tumors. Dashed lines indicate smaller subgroups of tumors with the respective diagnoses. *The majority of IDH-wild-type WHO grade II or III astrocytic tumors retain nuclear ATRX expression. IDH-wild-type WHO grade II or III astrocytomas are temporary entities in the 2016 WHO classification of tumors of the CNS. Adapted from (Reifenberger et al., 2016).

3.1.2 Characteristics of glioblastoma

Glioblastoma (WHO grade IV) accounts for the 45-50% of all gliomas, thus representing the most common primary malignant brain tumor. Its annual incidence in the United States (U.S.) is 3.2 new cases per 100 000 population. Several environmental factors have been analysed as a potential cause of glioblastoma development however ionizing radiation is the only accepted risk factor (Ostrom *et al.*, 2015). The 2016 CNS WHO classification incorporated distinction between (1) IDH-wild-type glioblastoma (90%), frequently defined as primary glioblastoma, predominating in patients over 50 years of age and (2) IDH-mutant glioblastoma (10%) which frequently develops from lower grade gliomas (secondary

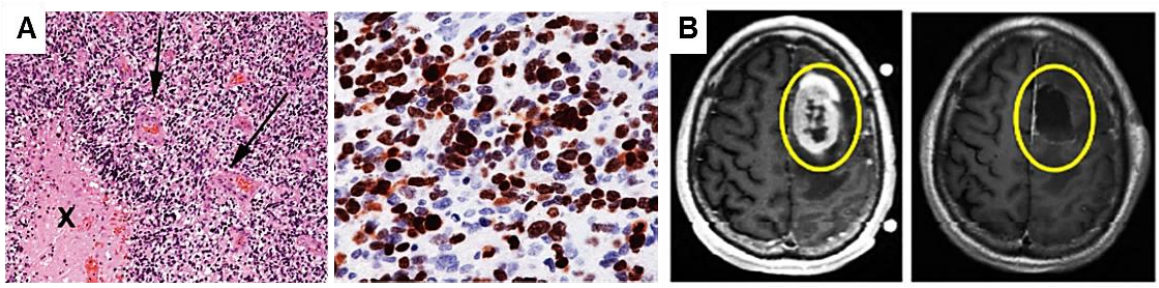
glioblastoma), commonly in younger patients. IDH-mutated glioblastoma is associated with longer clinical history (15 months) compared to the IDH-wild type (4 months) (Louis *et al.*, 2016). Although these two glioblastoma subtypes are histologically almost indistinguishable, they diverge at genetic and epigenetic level, with IDH-mutant glioblastomas displaying less necrosis and better prognosis (Ohgaki and Kleihues, 2013) (Table 1).

	IDH-wildtype glioblastoma	IDH-mutant glioblastoma
Synonym	Primary glioblastoma, IDH-wildtype	Secondary glioblastoma, IDH-mutant
Precursor lesion	Not identifiable; develops de novo	Diffuse astrocytoma Anaplastic astrocytoma
Proportion of glioblastomas	~90%	~10%
Median age at diagnosis	~62 years	~44 years
Male-to-female ratio	1.42:1	1.05:1
Mean length of clinical history	4 months	15 months
Median overall survival		
Surgery + radiotherapy	9.9 months	24 months
Surgery + radiotherapy + chemotherapy	15 months	31 months
Location	Supratentorial	Preferentially frontal
Necrosis	Extensive	Limited
TERT promoter mutations	72%	26%
TP53 mutations	27%	81%
ATRX mutations	Exceptional	71%
EGFR amplification	35%	Exceptional
PTEN mutations	24%	Exceptional

Table 1. Key characteristics of IDH-wild-type and IDH-mutant glioblastomas (Louis *et al.*, 2016).

The lesions are frequently unilateral, however, those localized in the brain stem and *corpus callosum* can involve both hemispheres and the latter termed “butterfly glioblastoma”.

Histopathology parameters such as nuclear atypia, cellular pleomorphism, high mitotic activity, microvascular proliferation and necrosis delineate glioblastomas (Ohgaki and Kleihues, 2013) (Fig. 2A). Although a prominent proliferative activity, detected by KI-67 expression is associated with glioblastoma, a correlation between the proliferation index and the clinical outcome of glioblastoma patients has not been confirmed (Moskowitz *et al.*, 2006). Glioblastomas infiltrate surrounding brain structures but metastasis is uncommon in patients without preceding surgical intervention. The anatomical localization and the tumor size define the clinical features of glioblastoma. Common symptoms include neurological disorders like hemiparesis and aphasia, behavioural and neurocognitive deficits. Magnetic resonance imaging (MRI) represents the conventional diagnostic tool for the examination of a patient with suspected or confirmed glioblastoma. Radiologically, glioblastomas appear as a ring-enhancing proliferative periphery zone around a non-enhancement necrotic centre (Nakada *et al.*, 2011) (Fig. 2B). Several factors are responsible for glioblastoma development and progression including deficiency in cell cycle control, up-regulation of growth factors and their receptors, angiogenic alterations, invasion and migration into surrounding tissue, aberrant apoptosis and genetic instability.



<u>Figure 2.</u> Histology and radiographic MRI images of glioblastoma. (A) Glioblastoma histopathological analysis showing (left) necrotic foci (x) which are typically surrounded by pseudopalisading tumor cells and microvascular proliferation (arrows) (hematoxylin & eosin staining; X100); (right) immunostaining for KI-67 (brown signal) delineates high mitotic activity (original magnification, X400). (B) T1-weighted MRI images of a glioblastoma before (left) and after (right) surgery. Yellow circles show the area of tumor (left) and the resection cavity after surgery (right). Adapted from (Preusser *et al.*, 2011; Redzic *et al.*, 2014).

3.1.3 Genetic and molecular profile of glioblastoma

The accumulation of genetic and epigenetic alterations drives malignant transformation towards glioblastoma. Mutations in the metabolic gene *IDH1* define a small subgroup of glioblastoma patients with favourable prognosis in the radiation and/or alkylating chemotherapy era (Table 1). Profiling of glioblastoma from the TCGA demonstrated that *IDH1* mutation correlates with a subset of tumors characterized by the CpG-island methylator phenotype (G-CIMP) (Noushmehr *et al.*, 2010), which results in transcriptional silencing of key regulatory genes (Jones and Baylin, 2007). IDH-wild-type glioblastomas are often associated with epidermal growth factor receptor (EGFR) mutation or amplification, phosphatase and tensin homologue (PTEN) mutation or deletion, homozygous deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A), amplification or loss of cyclin-dependent kinase 4 (CDK4), amplification of CDK6, mutation of retinoblastoma-associated protein (RB) or telomerase reverse transcriptase (TERT) promoter, methylation of O^6-methylguanine-DNA methyltransferase (MGMT), loss of chromosome 10 and gain of chromosome 7, genetic alterations in tumor protein p53 encoding gene (TP53), in ATRX as well as in genes encoding histone variant H3.3 (Ichimura *et al.*, 1998; Cen *et al.*, 2012; Ohgaki and Kleihues, 2013; Plass *et al.*, 2013) (Table 1 and Fig. 3).

EGFR

EGFR is a transmembrane glycoprotein which belongs to the ERBB family of receptor tyrosine kinases (RTK) (Zaczek *et al.*, 2005). Upon binding to one of the EGF family of ligands, EGFR transmits proliferative and pro-survival intracellular signals (Oda *et al.*, 2005). *EGFR* gene amplification and overexpression are found in 40% of IDH-wild-type glioblastomas. Amplification of the *EGFR* gene is frequently associated with the EGFR receptor variant III (EGFRvIII), which is characterized by a truncated extracellular domain, displaying constitutive ligand-independent activity (Wikstrand *et al.*, 1998). EGFRvIII confers tumorigenic potential to glioblastoma cells by promoting proliferative, pro-apoptotic and invasive signaling (Gan *et al.*, 2009).

RTK/PI3K/PTEN signaling pathway

The RTK/phosphoinositide 3-kinase (PI3K)/PTEN signaling cascade is aberrantly activated in glioblastoma (80% of the cases). This signaling cascade starts with the activation of a wide range of transmembrane RTK including EGFR or platelet-derived growth factor receptor (PDGFR) involved in recruitment of the cytoplasmic mediator PI3K and subsequent activation of downstream effector kinases such as protein kinase B (PKB/AKT) and mammalian target of rapamycin (mTOR). Abnormal activity in the RTK/PI3K/PTEN cascade has been observed in glioblastoma due to amplification or mutations in the key up-stream receptors controlling these molecules like *EGFR* and *PDGFR* or infrequently in the *PI3K* gene (less than 10% of the cases). Deletion or mutation in the tumor suppressor *PTEN* are observed in 40% of IDH-wild-type glioblastomas and associate with constitutive activation of PI3K/AKT pathway. Abnormal PI3K/AKT signalling results in disrupted regulation of several cellular processes including proliferation, migration, invasion, angiogenesis and activity of tumor suppressor pathways like p53 (Brennan *et al.*, 2013).

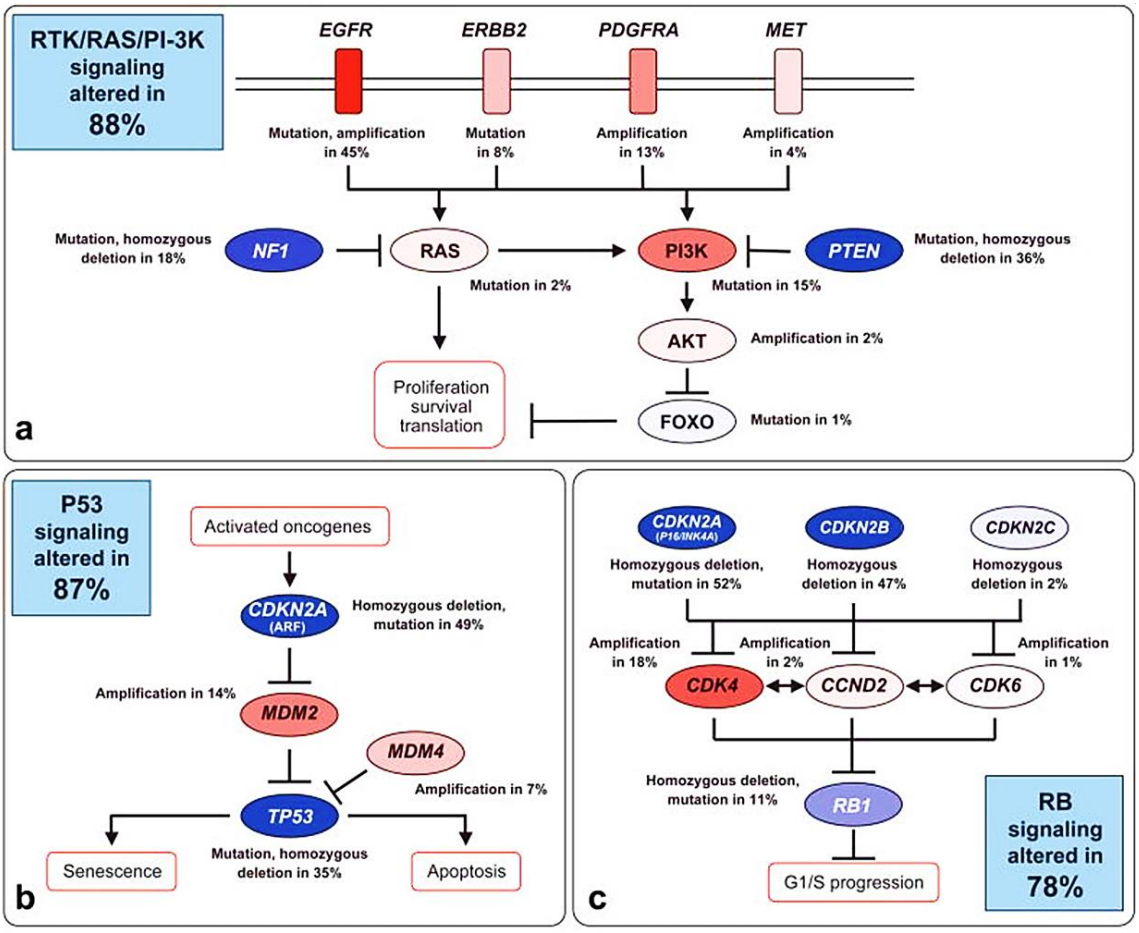
TP53/MDM2/p14ARF pathway

The *TP53* gene encodes for the p53 protein which is stimulated by different cellular stressors and triggers anti-proliferative signals (Vogelstein *et al.*, 2000). Aberrant p53 activity is frequently observed in IDH-mutant glioblastomas (90% of the case) but rare in IDH-wild-type glioblastomas (Watanabe *et al.*, 1996). Different events contribute to deregulating the p53 pathway. Amplification and overexpression of the p53 inhibitor, murine double minute 2 (MDM2), are also described as affecting mainly IDH-wild-type glioblastomas. Finally, loss of alternative reading frame protein 14 (p14ARF) expression, which binds and blocks MDM-2-dependent degradation of p53, is associate with the 50% and the 75% cases of IDH-wild-type and IDH-mutant glioblastoma, respectively (Nakamura *et al.*, 2001; Nakada *et al.*, 2011).

CDKN2A/CDK4/retinoblastoma

The tumor suppressor retinoblastoma-associated protein 1 (RB1) is a key regulator of the cell cycle and is alterated in around the 80% of glioblastomas. Once

phosphorylated by CDK4/cyclin D1 complex, RB1 mediates the release of E2 factor (E2F) and consequent induction of genes involved in the cell cycle progression. CDKN2A via inhibition of CDK4 prevents the G1-S transition. Thus, deletion in the *CDKN2A* locus, amplification of *CDK4* or loss of *RB1* are associated with the induction of E2F activity and therefore cell proliferation in glioblastoma (Cancer Genome Atlas Research, 2008; Ohgaki and Kleihues, 2009).

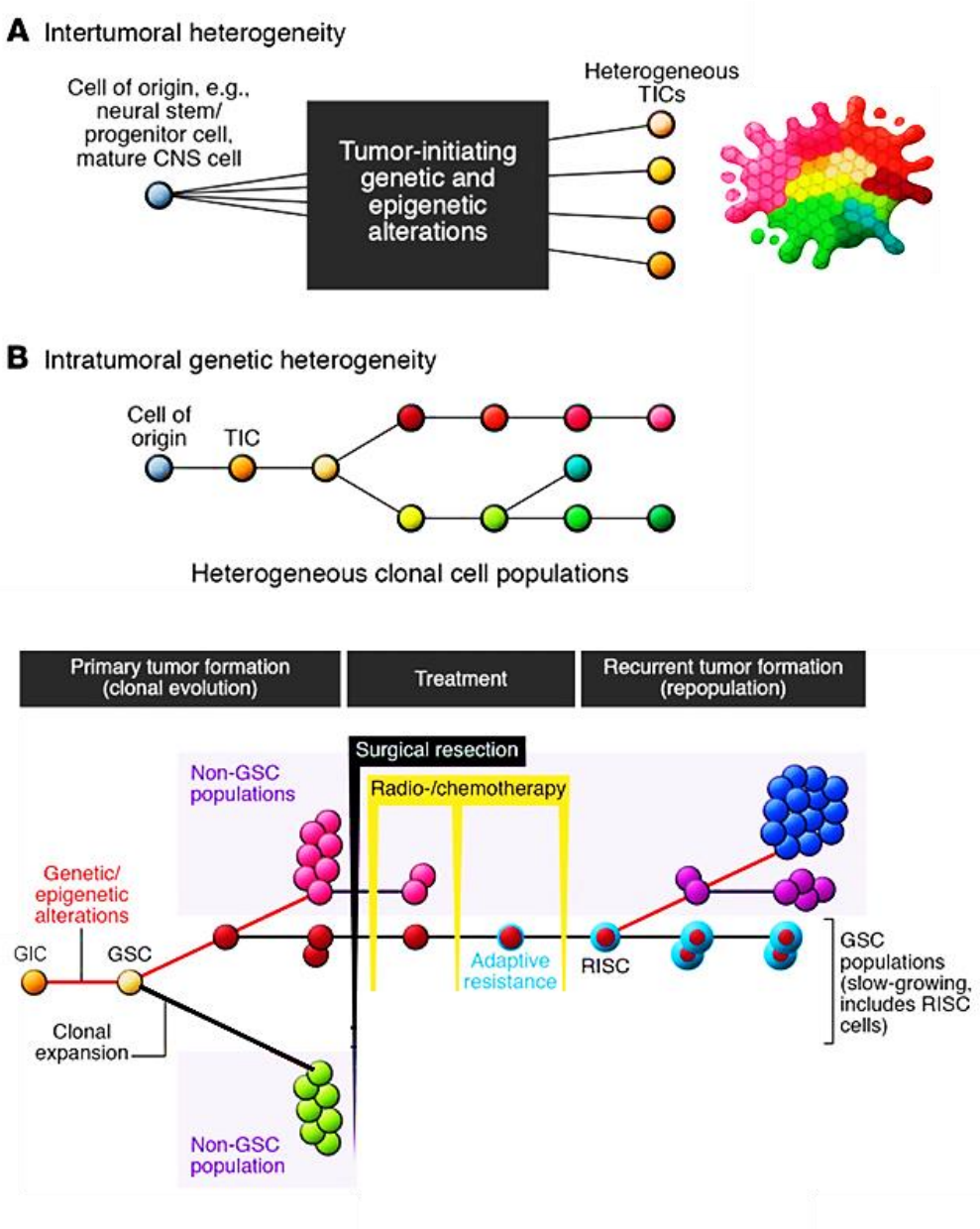


<u>Figure 3.</u> Genetic alterations associated with glioblastma. Summary of gene sequence modifications and copy number changes for components of the (A) RTK/RAS/PI3K, (B) p53 and (C) RB signaling pathways. Activating genetic alterations are labeled in red while inactivating alterations are labeled in blue. The type of mutation and the percentage of tumors affected are listed. The final percentages of glioblastomas displaying alterations in at least one gene of the designated pathway are indicated in boxes. Adapted from (Cancer Genome Atlas Research, 2008).

3.1.4 Origin of glioblastoma and glioma-initiating cells

The identification of the cells of origin of glioblastoma still represents a controversial field in glioblastoma research. A subset of tumor cells with stem-cell like properties, named cancer stem cells (CSC) or glioma-initiating cells (GIC), are currently considered as the driving force for glioblastoma development. This concept, designed as the CSC hypothesis, follows the idea that GIC originates from malignant transformation of neuroglial stem or progenitor cells (Singh *et al.*, 2004; Perez Castillo *et al.*, 2008). Alternatively, mature neural cells may also acquire mutations and dedifferentiate back to a deregulated stem-like phenotype (Garvalov and Acker, 2011) (Fig. 4A). The common anatomical localization of the tumors in the brain may support the CSC theory for glioblastoma. The subventricular zone (SVZ) of the lateral ventricle (LV) and the subgranular zone (SGZ) of the *dentate gyrus* (DG) represent the two main neurogenic regions where neural stem cells (NSC) and neural progenitors originate and may transform in GIC. GIC have been proposed to display self-renewal properties, multipotentiality and the capability to initiate a tumor with similar parental features after serial orthotopic transplantation in mice (Singh *et al.*, 2004; Vescovi *et al.*, 2006). When cultured in serum-free neurobasal media (NBM) supplemented with EGF and fibroblast growth factor (FGF) these cells grow as non-adherent spheres with the genetic profiles of the original glioblastoma specimen and with the potential to generate tumors upon intracranial xenografting (Singh *et al.*, 2004; Lee *et al.*, 2006; Ernst *et al.*, 2009). Fetal bovine serum inclusion in the culture media induces differentiation of GIC across different lineages (Singh *et al.*, 2003). Unlike NSC that permanently differentiate upon exposure to serum (Lee *et al.*, 2006), GIC differentiation is reversible and they can revert into undifferentiated spheres after serum deprivation (Qiang *et al.*, 2009). Defined markers commonly associated with NSC are shared and delineate the GIC population. Cluster of differentiation 133 (CD133), a glycoprotein expressed by hematopoietic cells and NSC, is the most frequently used cell surface marker for identification and isolation of GIC (Uchida *et al.*, 2000; Pfenninger *et al.*, 2007). However, the CD133 marker alone has been demonstrated to be not sufficient for a proper characterization of GIC; other stemness markers including NESTIN, sex determining region Y-box 2 (SOX-2), octamer-binding transcription factor-4 (OCT-4), Musashi-1, NANOG, integrin-α6, L1 cell adhesion

molecule (L1CAM) and CD15 are used for GIC identification (Persano *et al.*, 2013). GIC may be more resistant to irradiation and chemotherapy than non-stem cells, thus representing a challenging therapeutic target (Fig. 4B). Microenviromental factors and other multiple mechanisms have been defined as responsible for GIC maintenance and thus resistance to therapy, however, the molecular mechanisms controlling these processes remain poorly understood. GIC are localized within specific niches surrounding tumor vessels where by induction of abnormal vascular endothelial growth factor (VEGF) secretion as well as by differentiation into endothelial-like cells or pericytes (Ricci-Vitiani *et al.*, 2010; Wang *et al.*, 2010; Cheng *et al.*, 2013) they may promote angiogenesis and facilitate tumor growth (Bao *et al.*, 2006; Hamerlik *et al.*, 2012). Due to faster growth of tumor mass than tumor vasculature, broad necrotic regions develop near to the perivascular niche, generating hypoxic environment with high hypoxia-inducible factor 1-alpha (HIF-1α) levels which may also be involved in GIC maintenance (Persano *et al.*, 2013). Additionally, the hyperactivation of checkpoint pathways and increased MGMT protein levels promote repair of radiation-induced DNA damage rendering GIC resistant to therapies (Bao *et al.*, 2006; Hambardzumyan *et al.*, 2008; Pistollato *et al.*, 2010; Hsieh, 2011). The GIC population is also maintained by the upregulation of different signaling pathways involved in cell growth, differentiation and survival such as Notch, c-MET, transforming growth factor-β (TGF-β), nuclear factor-kappa B (NF-κB), WNT, EGF, signal transducer and activator of transcription-3 (STAT-3) and sonic hedgehog (shh) (Vescovi *et al.*, 2006). A better investigation of these signaling pathways might provide insights into the cellular features of GIC and improve treatments strategies for glioblastoma.



<u>Figure 4.</u> GIC contribute to glioblastoma heterogeneity and resistance to treatment. (A) GIC are considered as the cells of origin of glioblastoma. They may derive from neural stem, neural progenitors or differentiated CNS cells undergoing epigenetic and genetic alterations. GIC expand and acquire diverse genetic alterations generating a variety of clones responsible for tumor heterogeneity. (B) The primary tumor contains recurrence initiating stem-like cancer (RISC) cells (red circle). Initial treatments induce tumor regression, however, subpopulations of RISC cells survive therapeutic intervention though intrinsic and adaptive resistance mechanisms. RISC cells initiate tumor recurrence through a second round of clonal evolution that repopulates the tumor (blue circles). Adapted from (Osuka and Van Meir, 2017).

3.1.5 Heterogeneous nature of glioblastoma

Glioblastoma encompasses biologically distinct groups characterized by genetic and pathologic inter-tumor as well as intra-tumor heterogeneity (Sottoriva *et al.*, 2013; Inda *et al.*, 2014). The heterogeneous nature of glioblastoma can reflect the wide range of possible tumor cells of origin including NSC, astrocytes or oligodendroglial progenitor cells (Zong *et al.*, 2015; Osuka and Van Meir, 2017). Further diversity may derive from the subsequent acquisition of mutations in these cells during tumor progression.

Recent genomic approaches unveiled the intricacy of tumor heterogeneity in glioblastoma. Clonal heterogeneity within subsections of the same tumor reflects the glioblastoma evolutionary dynamics at the single patient level. Multiple sampling (primary tumor versus recurrent) from different regions of the tumor as well as single cell genomic technology paired with next generation sequencing analyses can be used to define the evolutionary events during glioblastoma development. Finally, these approches can be employed to define the markers of therapeutic resistance and tumor recurrence (Sottoriva *et al.*, 2013).

3.1.6 Treatment of glioblastoma

3.1.6.1 Standard of care for glioblastoma

Glioblastoma remains a fatal disease, with the majority of patients dying within 15-18 months and in less than 5% of cases after 5 years upon diagnosis (Stupp *et al.*, 2005; Stupp *et al.*, 2009; Weller *et al.*, 2014; Lu-Emerson *et al.*, 2015). The standard of care for glioblastoma consists of maximal surgical resection of the tumor mass followed by external beam radiation therapy at 60 Gy in 30-35 fractions and the concomitant administration of temozolomide (TMZ) (75 mg/m^2/day) followed by 6 maintenance cycles of TMZ (150-200 mg/m^2 in 5 days every 28 days) (Stupp *et al.*, 2005). TMZ is an orally active DNA alkylating agent of the imidazotetrazine class with lipophilic properties enabling its crossing of blood brain barrier (BBB). The surgical intent is to execute the largest tumor resection thus facilitating adjuvant therapy efficacy (Ryken *et al.*, 2008). The combinatorial treatment of radiotherapy plus TMZ has been shown to extend patient survival from 12 to 15 months (Stupp

et al., 2005; Huang *et al.*, 2016). TMZ exerts its therapeutic effect by methylation of DNA at different positions, e.g., the N-7 or O-6 positions of guanine and N-3 position of adenine to form N^7-methylguanine (N^7-MeG) (70%), O^6-methylguanine (O^6-MeG) (6%) or N3-methyladenine (N^3-MeA) (9%). Although less frequently observed, O^6-MeG constitutes the main DNA modification thought to mediate TMZ toxicity (Zhang *et al.*, 2011). These different DNA adducts induce DNA mismatch repair mechanisms (MMR) in response to DNA damage and therefore cell death (Sengupta *et al.*, 2012). Tumor cells influence the TMZ response by the expression of proteins with the ability to repair this type of DNA damage. Poly-adenosine diphosphate ribose polymerase (PARP) repairs N^7-MeG and N^3-MeG adducts, whereas MGMT repairs O^6-MeG lesions (Johnson and Chang, 2012). Approximately 35% of glioblastomas exhibit *MGMT* promoter methylation which results in epigenetic silencing of the gene with consequent impairment of DNA repair mechanisms and increased sensitivity to TMZ (Weller *et al.*, 2010). *MGMT* promoter methylation thus represents a predictive marker for the efficacy of chemotherapeutic agents in glioblastoma (Malmstrom *et al.*, 2012; Wick *et al.*, 2012). Other chemotherapeutic agents termed nitrosoureas, including carmustine (BCNU), lamustine (CCNU) or mimustine (ACNU) were used before TMZ and are currently considered as treatment options mainly for recurrent glioblastoma (Weller *et al.*, 2014).

3.1.6.2 Glioblastoma resistance to targeted therapies and innovative treatment strategies

Despite the standard multimodal therapeutic approach, glioblastoma recurrence is common and associated with poor prognosis. Negligible therapeutic success have been achieved over the past several decades and treatment options for relapsed glioblastoma remain limited (Weller *et al.*, 2015). Multiple factors are responsible for the therapeutic resistance and relapse in glioblastoma: (1) the presence of a patially intact BBB regulating drug delivery; (2) the highly invasive and heterogeneous nature of the tumor; (3) aberrant activation of DNA repair mechanisms; (4) the existence of a sub-population of cells with stem-like properties. More recently, the evaluation of novel therapeutic strategies has received increasing attention.

Glioblastoma is a highly vascularised tumor and expresses high levels of VEGF, the major angiogenic driver. Bevacizumab, a humanized monoclonal antibody against VEGF, has been approved by the U.S. FDA for recurrent glioblastoma in 2009 (Cohen *et al.*, 2009), but not in the European Union (E. U.). However, the results of two large randomized phase III trials - RTOG 0825 (Gilbert *et al.*, 2014) and AVAGlio (Chinot *et al.*, 2004), showed that bevacizumab together with standard therapy improves progression-free survival but not overall survival in newly diagnosed glioblastoma.

Thereutic approaches targeting EGFR have also been investigated for glioblastoma. Several clinical trials tested EGFR inhibitors including monoclonal antibodies (cetuximab), tyrosine kinase inhibitors (gefitinib or erlotinib) and EGFRvIII-targeted vaccines. Despite the discouraging results observed upon conclusion of these trials, EGFR, as a therapeutic agent in glioblastoma, is still under consideration (Reardon *et al.*, 2014). In recent years, novel immunotherapeutic approaches have been regarded as promising. Substantial work has shown that glioblastoma cells establish an immunosuppressive environment, which impairs T cell effector function. Targeting glioblastoma-induced immunosuppressive networks has been shown to significantly prolong survival in experimental glioma models, prompting further investigation in glioblastoma patients (Sampson *et al.*, 2014; Reardon *et al.*, 2016). Indeed, several clinical trials employing these molecules are ongoing (Weiss *et al.*, 2015), for instance, clinical trials using (i) immune checkpoint blockade by cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) inhibitors, (ii) genetically engineered, autologous cytotoxic T lymphocyte (CTL) therapy with vectors encoding T-cell receptors (TCR) or chimeric antibody receptors (CAR), (iii) autologous tumor lysate-pulsed dendritic cell vaccination (Prins *et al.*, 2013; Rushworth *et al.*, 2014; Lim *et al.*, 2016). Evidence from other tumor entities indicates that biomarker-based patient stratification increases immunotherapy efficacy (Schumacher *et al.*, 2015). Therefore, understanding which patients may benefit from a specific immunotherapy treatment may result in improved clinical responses in the near future. Other therapy modalities under investigation include gene therapy, peptide and dendritic cell vaccines, synthetic chlorotoxins, radiolabelled drugs and antibodies (Quang and Brady, 2004; Rich and Bigner, 2004; Mamelak *et al.*, 2006; Reardon *et al.*, 2006; Ferguson and Lesniak, 2007; Fulci and Chiocca, 2007).

In order to improve the design of targeted therapies in glioblastoma it might be necessary to better understand the dynamics of tumor progression, the role of different genetic alterations for response to therapy, the cross-talk between altered pathways as well as the network of interactions between tumor cells and the microenvironment. Additionally, molecular profiling of different tumor regions before and after therapy together with the analysis of tumour and/or circulating glioma cells from responders *versus* nonresponders patients might result in to the identification of biomarkers for individualized treatment (Adamczyk *et al.*, 2015; Reifenberger *et al.*, 2016).

3.2 Transforming growth factor (TGF)-β

3.2.1 Synthesis of the TGF-β superfamily of ligands

TGF-β ligands represent the prototypic molecules of a superfamily of interconnected cytokines containing more than 30 members in human (Lander *et al.*, 2001). The TGF-β superfamily of cytokines can be classified with respect to sequence similarities and function into two groups: (i) the TGF-β-like group that contains TGF-β, activin and nodal as well as (ii) growth and differentiation factors (GDF)/bone morphogenetic proteins (BMP)-like group constituted by BMP, GDF and anti-Mullerian hormone (AMH) (Shi and Massague, 2003). TGF-β ligands control several cellular processes such as morphogenesis, proliferation, differentiation, death, cytoskeletal organization, adhesion and migration (Massague, 2012). TGF-β is initially synthesized as a pre-pro-TGF-β monomeric 55 kDa precursor which is composed of a large amino-terminal signal peptide (SP), a region termed latency-associated peptide (LAP), and a carboxy-terminal portion containig the active form of TGF-β (Harrison *et al.*, 2011). SP cleavage and dimerization of two TGF-β monomers generates pro-TGF-β (Gentry *et al.*, 1988; Brunner *et al.*, 1989). Enzymes like furin convertase catalyze the cleavage of pro-TGF-β into the small latent TGF-β complex (SLC) comprised by two TGF-β chains connected with two LAP chains by non-covalent interactions (Rifkin, 2005; Todorovic *et al.*, 2005). A covalent binding of the latent TGF-β binding protein (LTBP) to the SLC yields the formation of the large latent TGF-β complex (LLC) (Gleizes *et al.*, 1996; Saharinen *et al.*, 1996; Saharinen and Keski-Oja, 2000; Chen *et al.*, 2005). LCC is then

secreted from cells into the extracellular space where the N-terminal region of LTBP forms covalent interactions with the extracellular matrix (ECM) and controls TGF-β availability (Fig. 5). The release of the LLC from the ECM and the delivery of mature TGF-β from the LAP are crucial steps for TGF-β functional activation and interaction with affiliate cell-surface receptors. The conversion into active TGF-β involves different proteases such as plasmin, matrix metalloproteinase -2/- 9 (MMP -2/-9), gelatinase, BMP-1, thrombospondin 1 (THBS1) that cleave LAP to release 25 kDa bioactive TGF-β (Sato and Rifkin, 1989; Flaumenhaft *et al.*, 1992; Kojima *et al.*, 1993; Schultz-Cherry *et al.*, 1994; Yu and Stamenkovic, 2000; Ge and Greenspan, 2006; Koli *et al.*, 2008). Conformational changes in the LLC bound to integrins represent another way by which mature TGF-β can be released (Wipff and Hinz, 2008). Three TGF-β mature ligands, TGF-β1, TGF-β2 and TGF-β3, have been reported in humans (Massague, 2000). These ligands share 70-80% structural homology but mediate different functions at least during development and their expression is different among tissues (Wu and Hill, 2009). *Tgf-β1* knock-out mouse models die due to defects in vasculature (Martin *et al.*, 1995). Ablation of mouse *Tgf-β2* results in embryonic death due to cardiac defects (Sanford *et al.*, 1997; Bartram *et al.*, 2001) and *Tgf-β3* knock-out mice have malformed cleft palate (Taya *et al.*, 1999).

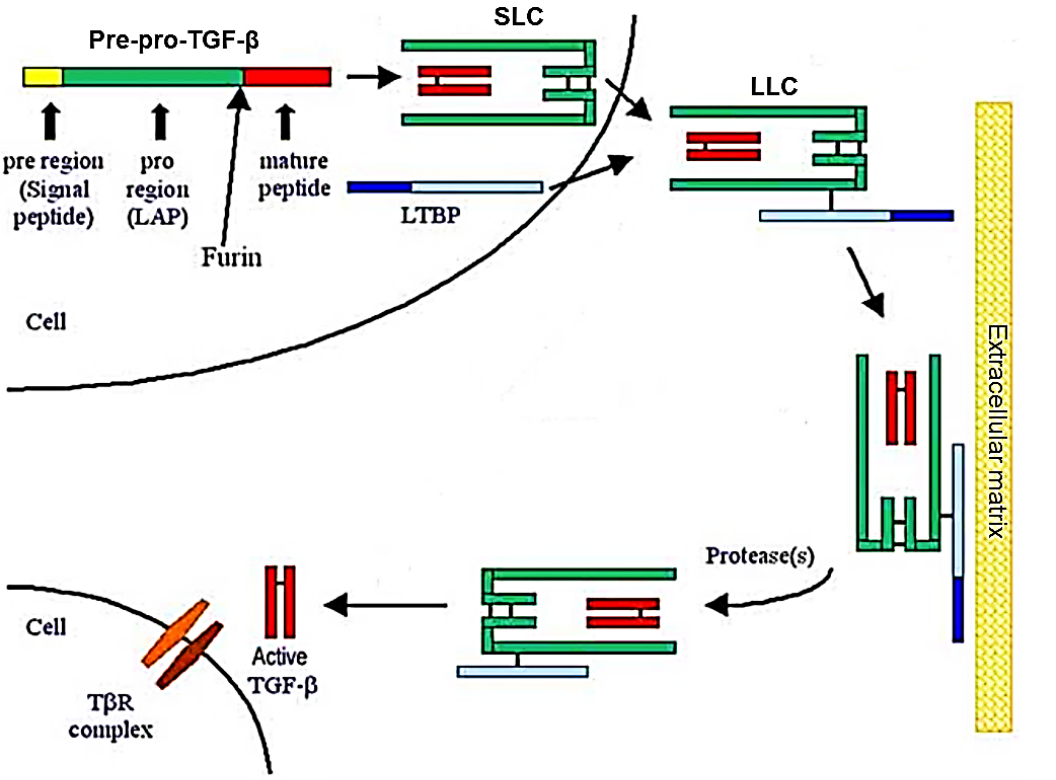


Figure 5. Illustration of the extracellular processing of latent TGF-β. TGF-β ligands are synthesized as part of a large latent TGF-β complex (LLC) formed by mature dimeric TGF-β and its latency-associate peptide (LAP) connected to a latent TGF-β binding protein (LTBP). Adapted from (Gressner *et al.*, 2002).

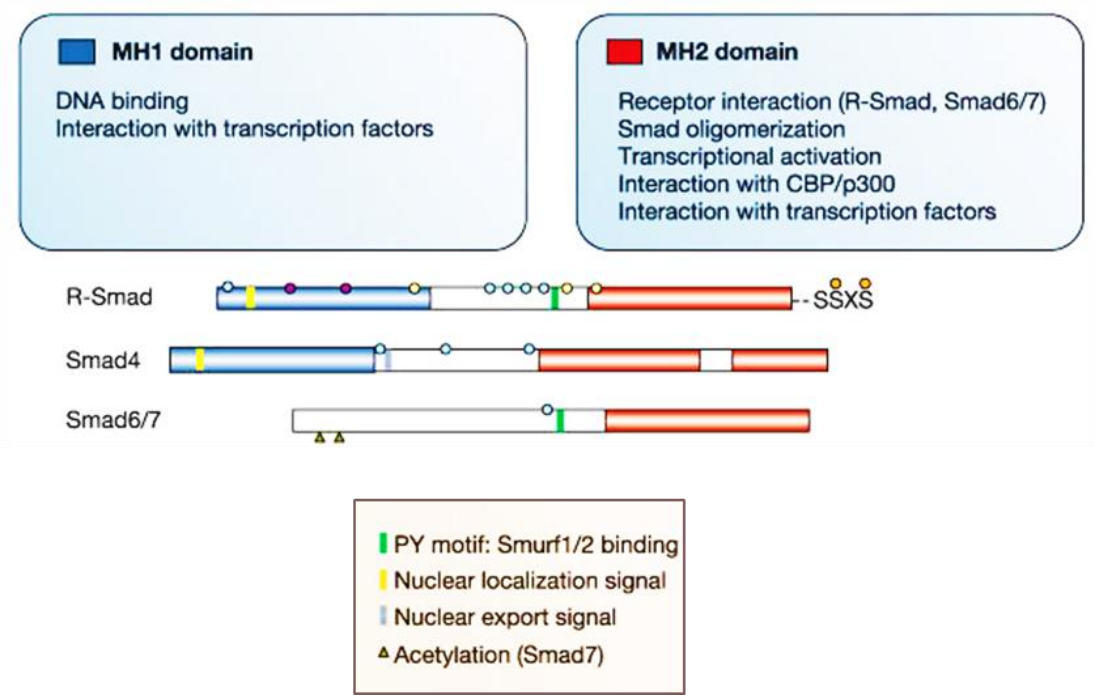
3.2.2 TGF-β family of receptors

TGF-β superfamily members signal by a heteromeric complex composed of TGF-β receptor type II (TGF-βRII) and type I (TGF-βRI) transmembrane serine/threonine kinase receptors. Five TGF-βRII and seven TGF-βRI have been found in humans. TGF-βRII and TGF-βRI are transmembrane glycoproteins containing an N-terminal ligand-binding extracellular domain linked by a transmembrane region to the C-terminal intracellular domain with the kinase activity (Okadome *et al.*, 1994; Hinck, 2012). TGF-βRII has a molecular weight of 75 kDa and is the product of a gene located on chromosome 3p22 (Lin *et al.*, 1992; Mathew *et al.*, 1994). TGF-βRI has a molecular weight of 53 kDa and is encoded by a gene located on chromosome 9q22 (Boyd and Massague, 1989; Pasche *et al.*, 1998). Although the ligand-receptor interactions have not been completely elucidated for all TGF-β superfamily ligands,

the formation of a heteromeric complex composed of two TGF-βRII, two TGF-βRI and a dimeric ligand is universal and widely accepted. This active receptor complex allows the constitutively active TGF-βRII to phosphorylate and thus activate the TGF-βRI on the GlySer (GS) domain (Wrana *et al.*, 1994; Manning *et al.*, 2002; Wakefield and Hill, 2013). Membrane-anchored co-receptors such as the TGF-β receptor type III (TGF-βRIII) (also known as betaglycan) and endoglin modulate the availability of TGF-β superfamily of ligands and add complexity to the regulation of ligand/receptor activation (Lopez-Casillas *et al.*, 1991; Bernabeu *et al.*, 2009). The seven different TGF-βRI are distinguished into two groups based on whether they transmit the signal to the TGFβ-like or BMP-like SMAD proteins (Weiss and Attisano, 2013). In addition to SMAD, TGF-β superfamily members transmit signals via non-canonical pathways including various branches of mitogen-activated protein kinase (MAPK) cascades, Rho-like GTPase signaling pathways and PI3K/AKT pathways.

3.2.3 Signaling pathways induced by the TGF-β superfamily

SMAD proteins are cytosolic mediators involved in the so-called TGF-β canonical signaling pathway. The human genome encodes eight different members of the SMAD family, which are functionally separated into three groups: receptor-activated SMAD (R-SMAD), common mediator SMAD (Co-SMAD) and inhibitory SMAD (I-SMAD) (Luukko *et al.*, 2001). The R-SMAD group comprises SMAD1/2/3/5/8 which are phosphorylated by the TGF-βRI; SMAD4 is a Co-SMAD that oligomerises with activated R-SMAD and allows their nuclear translocation. The I-SMAD group comprises SMAD6/7 which are also induced by TGF-β family members but negatively interfere with TGF-β signaling (Imamura *et al.*, 1997). SMAD proteins display two conserved domains, the N-terminal Mad homology 1 (MH1) and the C-terminal Mad homology 2 (MH2) domains (Massague *et al.*, 2005). The MH1 domain is conserved among R-SMAD and Co-SMAD but it is missing in I-SMAD. The MH1 domain is involved in the interactions with DNA and thus controls SMAD nuclear translocation and gene induction. All SMAD have a highly conserved MH2 domain which is involved in SMAD oligomerisation, recognition by TGF-βRI and interaction with adaptors and transcription factors (Fig. 6).



<u>Figure 6.</u> Structural and functional role of SMAD elements. SMAD proteins have MH1 and MH2 domains. The MH1 domain contains a β-hairpin structure for DNA binding. I-SMAD lack a MH1 domain. The linker region of SMAD4 contains a nuclear export signal. The MH2 basic pocket in SMAD interacts with activated TGF-βR. The MH2 basic pocket in SMAD4 allows the binding with the pS-x-pS motif (SxS) of R-SMAD (Derynck and Zhang, 2003).

TGF-β canonical signaling starts with ligand binding to the extracellular domain of the TGF-βRII and consequent transphosphorylation of TGF-βRI (Wrana *et al.*, 1994; Wakefield and Hill, 2013). The activated TGF-βRI recruits and phosphorylates the R-SMAD proteins. In general, the binding of TGF-β ligands promotes a characteristic combination of different TGF-βRII and TGF-βRI receptors on the plasma membrane (Shi and Massague, 2003; Massague *et al.*, 2005). TGF-β, activin and nodal induce the activation of a receptor complex composed of TGF-βRII or activin receptor 2 (ACVR2) and TGF-βRI (e.g. activin-receptor-like kinase ALK-4, ALK-5 and ALK-7) which signals via SMAD2/3. In contrast, SMAD1/5/8 are induced upon the binding of BMP ligands to a complex of receptors formed by BMP type II receptor (BMPRII) or ACVR2 and TGF-βRI (ALK1/2/3/6) (Fig. 7).

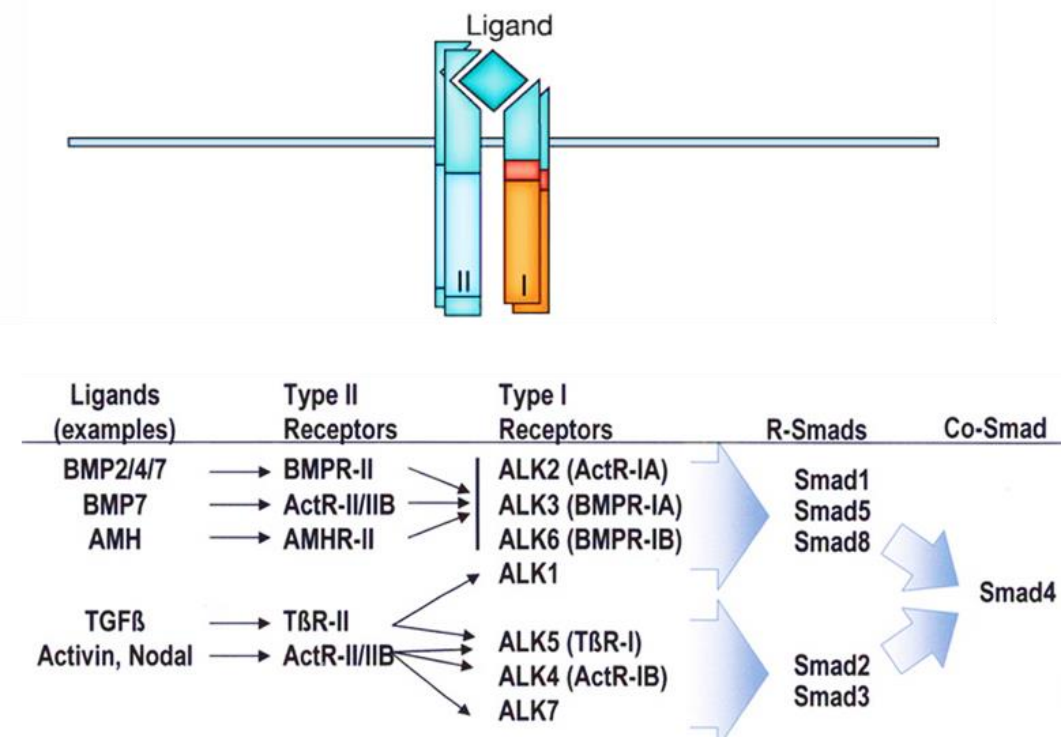
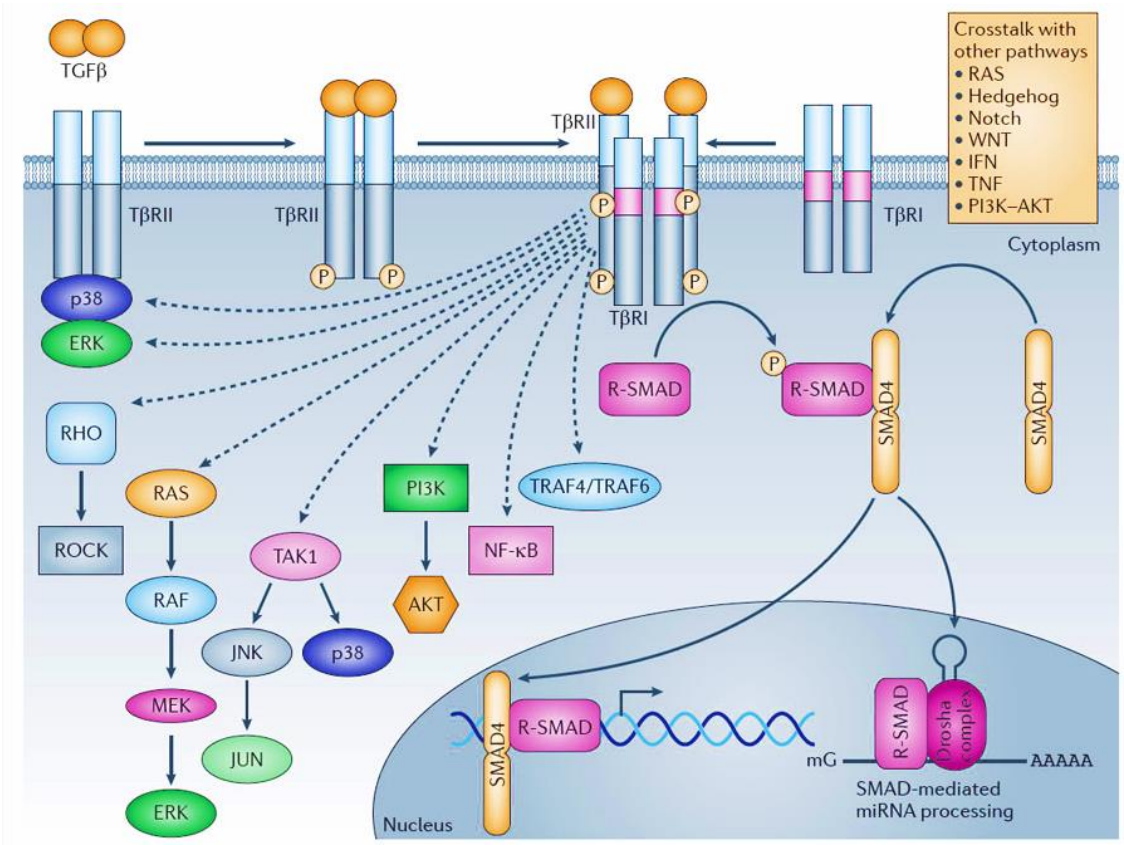


Figure 7. Schematic interactions of TGF-β family members with their specific Type II and Type I receptors as well as R-SMAD. Adapted from (Derynck and Zhang, 2003; Massague *et al.*, 2005).

Once phosphorylated, R-SMAD proteins detach from the receptor complex and associate with the common mediator SMAD4 within the cytoplasm (Massague, 1998; Lebrun *et al.*, 1999; Jayaraman and Massague, 2000). The R-SMAD/SMAD4 complex translocates into the nucleus where it recognizes the DNA sequence CAGAC, named SMAD binding element (SBE), and regulates specific target transcription. SMAD proteins bind with less affinity to some GC-rich sequences (Shi *et al.*, 1998). The association of SMAD with transcriptional coactivators or corepressors results in the induction or inhibition of their target gene (Massague *et al.*, 2005). I-SMAD execute a negative regulation of TGF-β signaling either by preventing phosphorylated R-SMAD/Co-SMAD interactions or by activation of SMAD specific E3 ubiquitin protein ligase (SMURF) (Imamura *et al.*, 1997; Nakao *et al.*, 1997; Hata *et al.*, 1998; Kavsak *et al.*, 2000; Itoh and ten Dijke, 2007). TGF-β/SMAD signaling regulates cell growth, differentiation, apoptosis, migration and invasion/metastasis (Proetzel *et al.*, 1995; Sanford *et al.*, 1997; Goumans and

Mummery, 2000; Massague, 2000). Alterations in the TGF-β canonical pathway occur in several human diseases, such as hereditary hemorrhagic telangectasia, fibrotic diseases, atherosclerosis, hereditary synostosis, hereditary chondrodysplasias, cleidocranial dysplasia and familial primary pulmonary hypertension (Akhurst and Hata, 2012). Additional diversity in TGF-β signaling is achieved via activation of the non-canonical SMAD-independent pathways. Non-SMAD signaling includes tumor necrosis factor (TNF) receptor-associated factor 4/6 (TRAF4/6), TGFβ-activated kinase 1 (TAK1), p38 MAPK, PI3K-AKT, extracellular signal-regulated kinase (ERK), JUN amino-terminal kinase (JNK) and NF-κB (Akhurst and Hata, 2012) (Fig. 8). Finally, WNT, Hedgehog, Notch, interferon, TNF and RAS pathways also contribute to the complexity of cellular responses generated by TGFβ superfamily members. The dynamic interconnection between canonical and non-canonical signaling cascades can affect the output of TGF-β signaling between growth suppression and induction of cellular plasticity (Massague and Gomis, 2006). In human cancer, TGF-β signaling exerts a dual role: in the early phase of tumorigenesis it may act as inhibitor of tumor development (Massague, 2000), but later on these suppressive functions are lost, and the TGF-β pathway turns to promote tumor progression, invasion, dissemination/metastasis, and immune evasion (Massague, 2008). Gene expression programmes controlled by TGFβ signaling pathways may provide tumor-suppressive or tumor-promoting functions depending on the tumor type and the stage of tumor progression (Akhurst and Hata, 2012). Thus, the functional outcome of the TGF-β response is context-dependent and determined by the cell, type and its state of activation.



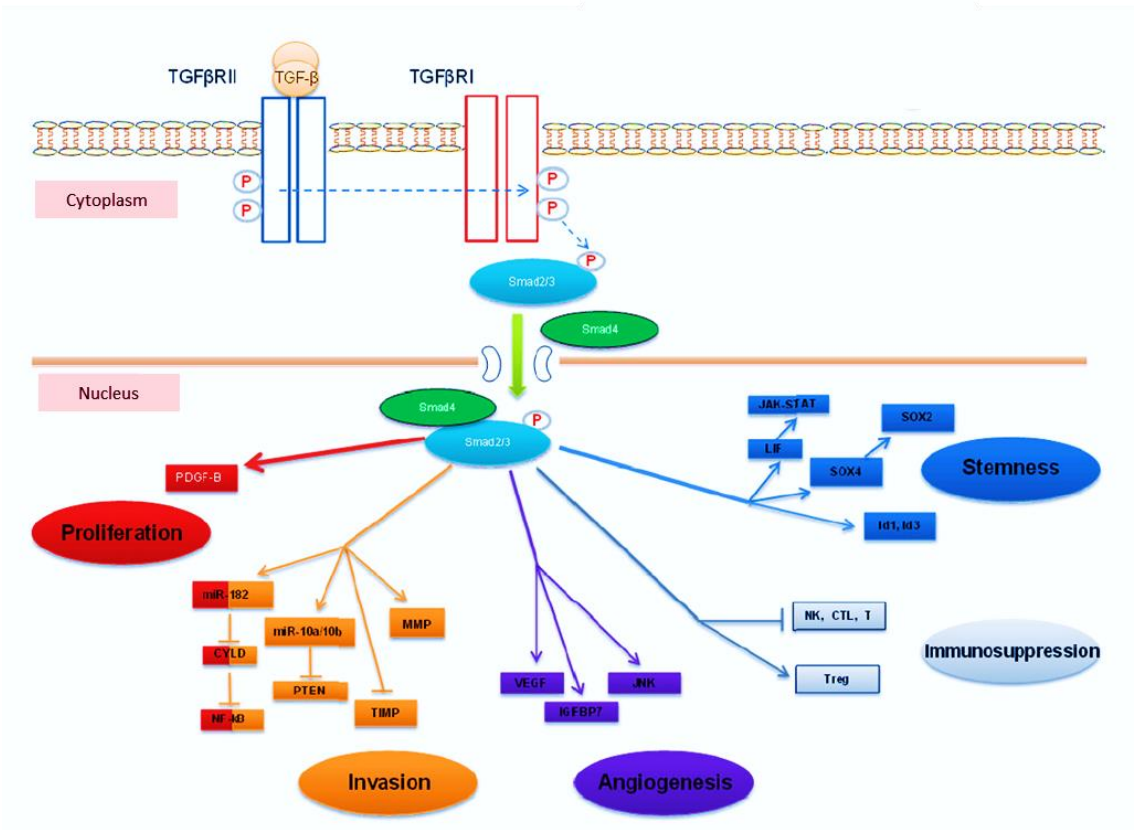
<u>Figure 8.</u> Schematic representation of TGF-β signaling pathways. Active TGF-β dimers induce the formation of TGF-β receptor type II (TβRII)/TGF-β receptor type I (TβRI) heteromeric complex. TβRII then transphosphorylates TβRI, which transmits intracellular signaling via R-SMAD phosphorylation. R-SMAD proteins interact with SMAD4 and translocate into the nucleus to regulate the expression of specific targets. In the non-canonical pathways, the activated TβR complex signals through other factors, such as TRAF4/6, TAK1, p38 MAPK, PI3K/AKT, ERK, JNK or NF-κB. TGF-β signaling can be influenced by other pathways than the canonical and non-canonical TGFβ signaling, such as WNT, Hedgehog, Notch, interferon, TNF and RAS pathways.

3.2.4 The role of TGF-β in glioblastoma

The TGF-β pathway is considered a crucial mediator of glioblastoma pathogenesis due to its control of cell proliferation, tumor invasion, angiogenesis, immunosuppression and the maintenance of GIC population (Ueki *et al.*, 1992; Alexandrow and Moses, 1995; Wesolowska *et al.*, 2008; Ikushima *et al.*, 2009) (Fig.

9). Glioblastoma patients show high level expression of TGF-β which has been linked to poor prognosis (Bruna *et al.*, 2007; Hau *et al.*, 2011). Recent data from our laboratory, however, challenge this view (Frei *et al.*, 2015). PDGF-B upregulation by aberrant TGF-β/SMAD signaling may lead to tumor cell proliferation (Seoane *et al.*, 2004; Bruna *et al.*, 2007). TGF-β promotes cell invasion by increasing the expression of MMP or suppressing tissue inhibitors of metalloproteinase (TIMP) (Wick *et al.*, 2001). Additionally, TGF-β upregulates micro RNA (miR) -10a/-10b expression, which may promote migration of glioma cells via inhibition of PTEN (Liu *et al.*, 2013). The pro-angiogenic effect of TGF-β arises from its stimulatory effect on the expression of angiogenic factors including VEGF, FGF and plasminogen activator inhibitor (PAI-1) (Dieterich *et al.*, 2012; Mangani *et al.*, 2016). In this context, TGF-β signaling induces insulin-like growth factor-binding protein 7 (IGFBP7), a tumor vessel biomarker associated with glioblastoma (Pen *et al.*, 2008). TGF-β and other tumor-secreted cytokines generate an immunosuppressive glioblastoma microenvironment through a variety of mechanisms (Zhu *et al.*, 2012). For instance, TGF-β2 decreases human leukocyte antigen D related (HLA-DR) promoting immune escape of tumor cells from T cell attack (Zuber *et al.*, 1988). Moreover, TGF-β inhibits the expression of perforin, granzyme A, granzyme B, Interferon-γ, and Fas ligand, which are co-responsible for cytotoxic T lymphocyte (CTL)-mediated tumor cytotoxicity (Thomas and Massague, 2005). Immunosuppressive regulatory T (T-reg) cells and cytokine interleukin-10 (IL-10) expression are also promoted by TGF-β (Maeda *et al.*, 1995; Chen *et al.*, 2005). Furthermore, TGF-β immunosuppressive function includes a reduction of the activating immune cell receptor natural killer group 2, member D (NKG2D) in CD8+ T cells and natural killer (NK) cells, reducing the lytic activity of these cells against glioma cells (Friese *et al.*, 2004; Ikushima *et al.*, 2009). TGF-β may be more abundant in GIC than undifferentiated glioma cells (Qiu *et al.*, 2011). High TGF-β signaling endorses DNA damage responses in GIC thus rendering this population resistant to DNA damaging agents (Hardee *et al.*, 2012). TGF-β is also involved in the induction of stem cell marker essential for GIC maintenance. Inhibition of TGF-β signaling was shown to be associated with the decrease in the expression of *SOX-2* with consequent reduction of GIC stem-like properties (Ikushima *et al.*, 2009). Moreover, TGF-β-SMAD signaling can up-regulate the leukemia inhibitory factor (LIF) leading to the activation of the JAK-STAT signaling pathway involved in self-

renewal capacity of GIC (Penuelas *et al.*, 2009). Finally, the inhibition of TGF-βRI abrogates the ability to initiate tumors in the CD44 h^{igh}/DNA binding protein inhibitor (Id1) ^{high} GIC population (Anido *et al.*, 2010).



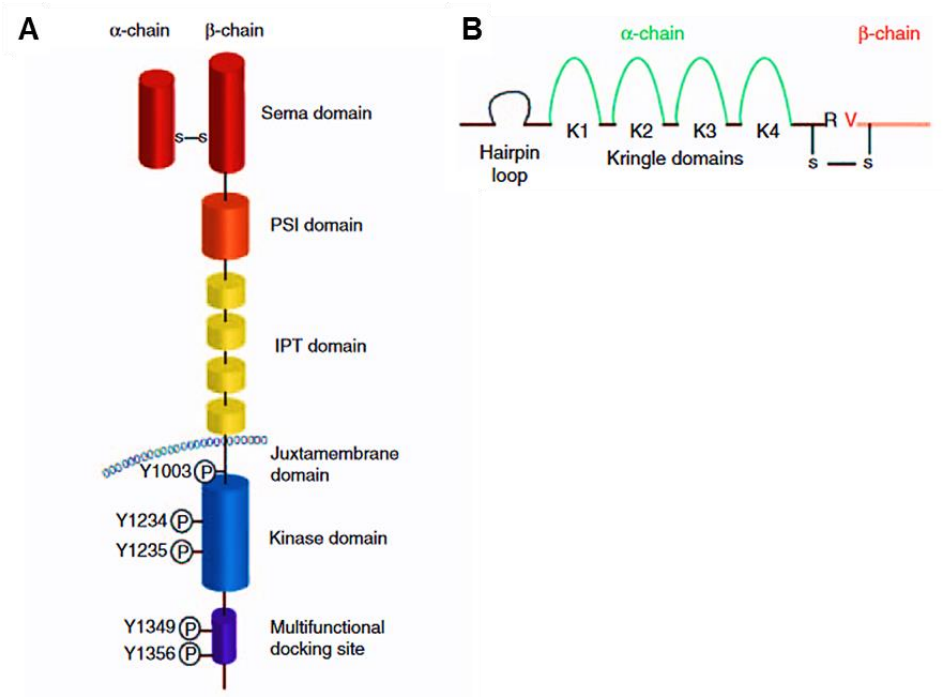
<u>Figure 9.</u> TGF-β signaling in gliomagenesis. The TGF-β pathway promotes glioblastoma via induction of different pro-tumorigenic signals: proliferation via PDGF-B and miR-182; invasiveness via miR-182, miR-10 and MMP; angiogenesis via VEGF, IGFBP7 and JNK. TGF-β exerts an immunosuppressive role by inhibiting NK cells, CTL, dendritic cells (DC), and by T-reg cells. In addition, the TGF-β pathway maintains the GSC population via LIF, SOX-4/SOX-2 axis, and ID1-ID3. Adapted from (Han *et al.*, 2015).

3.3 Hepatocyte growth factor (HGF)/c-MET

3.3.1 Structure and functions of HGF and its receptor c-MET

c-MET is a cell surface RTK encoded by a gene located on chromosome 7q21-31. Its expression is reserved to epithelial cells of different organs, including liver, pancreas, prostate, kidney, muscle and bone marrow, both during embryogenesis and adulthood (Comoglio *et al.*, 2008). c-MET is produced upon proteolytic processing of its precursor in the post-Golgi compartment. Mature c-MET receptor consists of a single-pass α/β heterodimer composed by a completely extracellular α-subunit linked by a disulphide bond to a transmembrane β-subunit displaying intracellular catalytic activity (Fig. 10A) (Trusolino and Comoglio, 2002). The extracellular part of c-MET includes three functional domains: the semaphorin (sema) domain, which encompasses the whole α-subunit and part of the β-subunit, the plexin-semaphorin-integrin (PSI) domain composed by four disulphide bonds and four immunoglobulin-plexin-transcription (IPT) domains. The intracellular segment is formed by three domains: a juxtamembrane sequence that negatively controls the kinase activity when phosphorylated on Ser975, a catalytic region that positively controls kinase activity upon trans-phosphorylation of Tyr1234/1235 and a carboxy-terminal multifunctional docking site that contains two tyrosines (Tyr1349/1356) involved in the recruitment of several transducers and adaptors (Trusolino *et al.*, 2010). HGF, also known as scatter factor, is the ligand of c-MET (Stoker *et al.*, 1987; Nakamura, 1989; Weidner *et al.*, 1991). HGF broadly distributes in the ECM of different tissues where is sequestered in its inactive form by heparin-like proteoglycans (Kobayashi *et al.*, 1994; Lyon *et al.*, 1994). The principal sources of HGF secretion are mesenchymal cells. HGF is produced as a biologically inactive single-chain precursor further converted into a disulphide linked α/β chains active heterodimer by extracellular proteases (Fig. 10B). The α-chain contains an amino-terminal hairpin loop (HL) and four kringle domains (k1-k4). The β-chain is formed of a serine protease homology (SPH) domain without proteolytic activity. HGF is a pleiotropic cytokine, involved in induction of cell proliferation, survival, motility, scattering, differentiation and morphogenesis (Trusolino and Comoglio, 2002; Birchmeier *et al.*, 2003; Basilico *et al.*, 2008). The cell-scattering phenotype, a key epithelial function during embryogenesis and wound repair, is characterized by

destruction of the cadherin-based cell-cell contacts and consequent cell motility (Corso *et al.*, 2005). This process is also crucial for the long-range migration of skeletal muscle progenitor cells during embryogenesis. Indeed, mutant mice with ablation HGF- or c-MET-dependent signals exhibit different deficits that are related to reduction in proliferative signals. *c-Met* or *Hgf* knockout mouse embryos show significant reduction in liver size. Moreover, abnormal placental development in *Hgf* and *c-Met* knockout mice leads to death of the embryos in utero (Schmidt et al., 1995; Uehara et al., 1995).



<u>Figure 10.</u> Domain structure of c-MET and HGF. (A) c-MET is synthesized upon proteolytic processing of a precursor into a single-pass, disulphide-linked α/β heterodimer. The extracellular portion of c-MET is composed of three domain types. The N-terminal is formed by a SEMA domain, the PSI domain and four IPT domains. The C-terminal part contains a tyrosine kinase catalytic domain flanked by juxtamembrane and carboxy-terminal sequences. The Y1234/1235 residues on the kinase catalytic domain positively modulate the enzyme activity, while the juxtamembrane Y1003 negatively regulates c-MET. Y1349/1356 tyrosines are multifunctional docking sites recruiting transducers and adaptors. (B) HGF is produced as a single-chain precursor and then converted into its active form upon cleavage of the bond between Arg494 and Val495 by extracellular proteases. Mature HGF is composed of a disulphide linked α/β-chain. Adapted from (Organ and Tsao, 2011).

3.3.2 HGF/c-MET signaling pathway

HGF/c-MET signaling is initiated by the binding of HGF to the amino-terminal region of c-MET and consequent receptor homodimerization and phosphorylation on the two Y1234/1235 residues (Rodrigues and Park, 1994) (Fig. 11). This is followed by the phosphorylation of two additional Y1349/1356 residues of the c-MET carboxy-terminal tail (Ponzetto *et al.*, 1994) and the recruitment of several effector molecules such as: growth factor receptor-bound protein 2 (GRB2) (Fixman *et al.*, 1996), src homology 2 domain-containing (SHC) (Pelicci *et al.*, 1995), v-crk sarcoma virus CT10 oncogene homolog (CRK), CRK-like (CRKL) (Garcia-Guzman *et al.*, 1999; Sakkab *et al.*, 2000), PI3K, phospholipase Cγ (PLCγ), v-src sarcoma (Schmidt *et al.*, 1999) viral oncogene homolog (SRC) (Ponzetto *et al.*, 1994), src homology 2 domain-containing phosphatase 2 (SHP2) (Schaeper *et al.*, 2000; Maroun *et al.*, 2003) and STAT-3 (Boccaccio *et al.*, 1998; Zhang *et al.*, 2002). Unique for c-MET is the binding of the multiple adaptor protein termed GRB2-associated binding protein 1 (GAB1) (Weidner *et al.*, 1996). GAB1 associates either directly or through GRB2 to phosphorylated c-MET and allows the formation of binding sites for more downstream adaptors. This machinery activates different downstream signal transduction pathways that include MAPK cascades, JNK, p38, PI3K-AKT axis, focal adhesion kinase (FAK) and the inhibitor of NF-κB (IκBα)/NF-κB complex (Maroun *et al.*, 1999; Sipeki *et al.*, 1999; Maroun *et al.*, 2000; Lai *et al.*, 2009). The sum of these signaling pathways controls different processes such as cell proliferation, survival, motility, scattering, differentiation and morphogenesis (Trusolino and Comoglio, 2002; Birchmeier *et al.*, 2003; Basilico *et al.*, 2008). Different signals play a negative control for c-MET activation. c-MET phosphorylation on the Y1003 residue has an inhibitory effect on the c-MET pathway (Peschard *et al.*, 2001; Petrelli *et al.*, 2002). Several protein tyrosine phosphatases (PTP) are involved in c-MET dephosphorylation and its consequent inactivation (Palka *et al.*, 2003; Machide *et al.*, 2006). Finally, protein kinase C (PKC) blocks c-MET activity (Gandino *et al.*, 1990; Gandino *et al.*, 1994). Increased levels of intracellular calcium can also have a negative effect on c-MET (Gandino *et al.*, 1991). Additional complexity is generated by the association of c-MET with co-receptors at the cell surface, influencing the outcome of c-MET dependent signalling (Trusolino *et al.*, 2010).

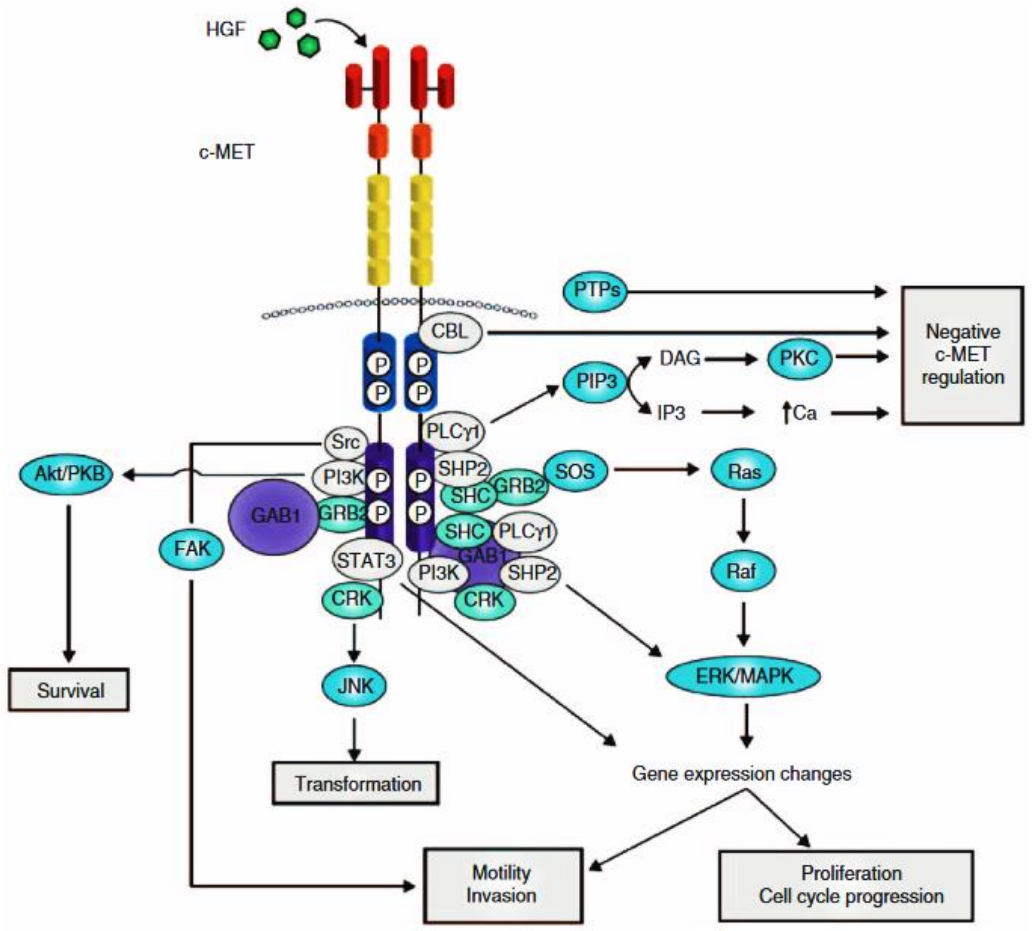


Figure 11. c-MET signaling adaptors and mediators. Active c-MET recruits signaling effectors including GRB2, SHC, CT10, CRK and CRKL and the effector molecules PI3K, PLCγ, SRC, SHP2 and STAT-3. In addition, the multi-adaptor protein GAB1 bound to phosphorylated c-MET forms binding sites for other downstream adaptors. The downstream response to c-MET activation is similar to many RTK and include cell proliferation, survival, motility, scattering, differentiation and morphogenesis. Adapted from (Organ and Tsao, 2011).

3.3.3 HGF/c-MET signaling in glioblastoma

Although genetic alterations in *c-MET* rarely occur in glioblastoma (4%), a crucial role for the c-MET pathway has been attributed in glioblastoma pathogenesis since c-MET is often overexpressed together with its ligand (Koochekpour *et al.*, 1997). HGF and c-MET are also expressed by endothelial cells and may contribute to glioblastoma neoangiogenesis. Aberrant activation of c-MET promotes proliferation

in glioblastoma via activation of c-MYC-dependent G1/S cell cycle progression (Walter *et al.*, 2002). c-MET prevents DNA damage-induced cell death and promotes survival of glioblastoma cells (Bowers *et al.*, 2000). The role of HGF/c-MET in glioblastoma invasive growth is well recognised (Brockmann *et al.*, 2003). Invasion and migration of glioblastoma cells are supported by HGF dependent induction of MMP-2 and urokinase-type plasminogen activator (uPA) expression (Hamasuna *et al.*, 1999). In addition, c-MET activity has been associated with glioblastoma stem-like properties, including glioblastoma resistance to therapies and tumor progression (Joo *et al.*, 2012). Active c-MET up-regulates the expression of a panel of transcription factors, including SOX-2, c-MYC, kruppel-like factor-4 (KLF-4), OCT-4, and NANOG, involved in reprogramming differentiated cells into pluripotent stem cells (Li *et al.*, 2011). Several studies support a role for c-MET in the regulation of the glioblastoma stem cell phenotype and described a correlation between c-MET levels and tumorigenic potential of glioblastoma (De Bacco *et al.*, 2012; Joo *et al.*, 2012).

3.4 Targeting TGF-β or c-MET pathways for glioblastoma therapy

To date, there are 10 different registered trials targeting TGF-β or HGF/c-MET pathways in glioblastoma patients (Table 2). Table 2 lists the summary of (A) TGF-β and (B) HGF/c-MET clinical trials. Due to the high level of complexity characterizing glioblastoma, only a subset of patients may respond to these inhibitory strategies. It is therefore crucial to elucidate biomarkers defining sensitivity to TGF-β or c-MET targeting in glioblastoma. The current research knowledge supports the combination of TGF-β or HGF/c-MET blockade with cytotoxic therapies such as radiotherapy and chemotherapy or anti-angiogenic therapies. In addition, interconnections among the multiple pathways and the presence of compensatory mechanisms lead to treatment resistance in glioblastoma. The use of combination therapies that concurrently target multiple pathways are likely to reach superior therapeutic efficacy. However, the interactions between oncogenic pathways remain poorly understood, and further studies are needed for defining novel and more efficient combination therapies in glioblastoma.

A Clinical trials targeting TGF-β pathway in glioblastoma

Rank	Status	Study
1	Completed	Phase IIb Clinical Trial With TGF-β2 Antisense Compound AP 12009 for Recurrent or Refractory High-grade Glioma **Conditions:** Glioblastoma; Anaplastic Astrocytoma **Interventions:** Drug: AP 12009 10 µM; Drug: AP 12009 80 µM; Drug: temozolomide or PCV; Device: Drug delivery system for administration of AP 12009; Procedure: Placement of Drug Delivery System
2	Recruiting	A Study of Galunisertib (LY2157299) in Combination With Nivolumab in Advanced Refractory Solid Tumors and in Recurrent or Refractory NSCLC, Hepatocellular Carcinoma, or Glioblastoma **Conditions:** Solid Tumor; Non-Small Cell Lung Cancer Recurrent; Hepatocellular Carcinoma Recurrent; Glioblastoma **Interventions:** Drug: Galunisertib; Drug: Nivolumab
3	Completed	A Study Combining LY2157299 With Temozolomide-based Radiochemotherapy in Patients With Newly Diagnosed Malignant Glioma **Condition:** Glioma **Interventions:** Drug: LY2157299; Drug: Radiation; Drug: Temozolomide
4	Completed	Safety and Imaging Study of GC1008 in Glioma **Condition:** Primary Brain Tumors **Interventions:** Other: 89Zr-GC1008; Drug: GC1008
5	Terminated Has Results	Efficacy and Safety of AP 12009 in Patients With Recurrent or Refractory Anaplastic Astrocytoma or Secondary Glioblastoma **Conditions:** Anaplastic Astrocytoma; Glioblastoma **Interventions:** Drug: trabedersen; Drug: temozolomide; Device: Drug delivery system for administration of AP 12009; Procedure: Placement of Drug Delivery System; Drug: carmustine; Drug: lomustine

B Clinical trials targeting HGF / c-MET pathway in glioblastoma

Rank	Status	Study
1	Completed	A Study of Onartuzumab (MetMAb) in Combination With Bevacizumab Compared to Bevacizumab Alone or Onartuzumab Monotherapy in Participants With Recurrent Glioblastoma **Condition:** Glioblastoma **Interventions:** Drug: Bevacizumab; Drug: Onartuzumab; Drug: Placebo
2	Recruiting	INC280 Combined With Bevacizumab to Evaluate Glioblastoma Multiforme, Metastatic Colorectal Cancer, and Metastatic Renal Cell Carcinoma Patients **Conditions:** Glioblastoma Multiforme; Gliosarcoma; Colorectal Cancer; Renal Cell Carcinoma **Interventions:** Drug: INC280; Biological: bevacizumab
3	Completed	Safety and Efficacy of INC280 and Buparlisib (BKM120) in Patients With Recurrent Gliobastoma **Condition:** c-MET Inhibitor; PI3K Inhibitor, PTEN Mutations, Homozygous Del. of PTEN or PTEN Neg. by IHC, c-Met Ampli. by FISH, INC280, BKM120, Buparlisib; Recurrent GBM **Interventions:** Drug: INC280; Drug: Buparlisib
4	Withdrawn	A Study Of Crizotinib Plus VEGF Inhibitor Combinations In Patients With Advanced Solid Tumors. **Conditions:** Carcinoma, Renal Cell; Glioblastoma; Carcinoma, Hepatocellular **Interventions:** Drug: Crizotinib plus VEGF inhibitor combinations; Drug: Crizotinib plus axitinib; Drug: Crizotinib plus sunitinib; Drug: Crizotinib plus bevacizumab; Drug: Crizotinib plus sorafenib
5	Active, not recruiting	Study of INC280 in Patients With c-MET Dependent Advanced Solid Tumors **Condition:** Solid Tumors **Intervention:** Drug: INC280

Table 2. List of the clinical trials targeting (A) TGF-β or (B) HGF/c-MET signaling pathways in glioblastoma. Data from www.clinicaltrials.gov.

4. Materials and Methods

Reagents

EMD1214063 is a highly specific, reversible and ATP-competitive, small molecule c-MET tyrosine kinase inhibitor developed by Merck Serono (Darmstadt, Germany) (Bladt *et al.*, 2013). SD-208 (Scios Inc., Sunnyvale, CA) is a TGF-βRI (ALK-5) inhibitor (Uhl *et al.*, 2004). Galunisertib (LY2157299 monohydrate) is a TGF-βRI (ALK-5) inhibitor (Selleckchem, Houston, TX). U0126 is a highly selective inhibitor of MEK1 and MEK2 (Cell Signaling Technology, CST Denvers, MA). AZD5363 is a serine/threonine protein kinase and protein kinase B alpha inhibitor (AstraZeneca, Cheshire, UK). All drugs were dissolved in dimethylsulfoxide (DMSO) and further diluted in cell culture medium (final solvent concentration <0.001%). Recombinant human HGF, human TGF-β1 and TGF-β2 (R&D Systems, Minneapolis, MN) were dissolved in Dulbecco's phosphate-buffered saline (PBS).

Cell culture

ZH-161, ZH-305 and T-269 GIC lines were isolated from surgically removed *de novo* glioblastomas (Lemke *et al.*, 2014; Seystahl *et al.*, 2015). Glioblastoma tissue samples were washed with Hanks' balanced salt solution (Invitrogen, Carlsbad, CA), and digested with 1 mg ml^{-1} collagenase/dispase (Roche, Mannheim, Germany) for 30 min at 37 °C. After digestion, the fragments were filtered using a 70 µm cell mesh (Sigma-Aldrich, St Louis, MO), and then 2,500-5,000 cells were plated into T25 flasks. The cells were cultured in neurobasal medium (NBM) supplemented with 2% B27, 1% glutamine, EGF (10 ng/ml) and basic FGF (20 ng/ml). Spheres with diameters from 200-500 µm were dissociated mechanically for further passaging. The cell lines are routinely evaluated for self-renewal capacity, stem cell marker expression, and *in vivo* tumorigenicity in nude mice.

Real-time polymerase chain reaction (RT-PCR)

Total mRNA extraction was performed using the NucleoSpin®RNA II system including DNase treatment (Macherey-Nagel, Düren, Germany). cDNA was prepared using the iScript™ cDNA synthesis kit (Biorad Laboratories, Reinach, Germany) and random primer 9 (New England Biolabs, Ipswich, MA). For RT-PCR,

gene expression was measured using SYBR Green chemistry (AppliChem, Darmstadt, Germany) with the Real Time PCR System 7300 (Applied Biosystems, Foster City, CA). The conditions for RT-PCR were 40 cycles, 95°C/15 sec, 60°C/1 min. Specific target gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1). The following primers were used: HPRT1 (forward 5'-TGA GGA TTT GGA AAG GGT GT-3', reverse 5'-GAG CAC ACA GAG GGC TAC AA-3'); HGF (forward 5'-CCG AAC AGG ATT CTT TCA CC-3', reverse 5'-AGG AGG AGA TGC AGG AGG AC-3'); c-MET (forward 5'-ATG GAA ATG CCT CTG GAG TG-3', reverse 5'-CTG AGA TAC GCA GCC TGA AG-3'); OCT-4 (forward 5'-CGA GAA GGA TGT GGT CCG AG-3', reverse 5'-TGT GCA TAG TCG CTG CTT GA-3'); SOX-2 (forward 5'-CAC ACT GCC CCT CTC AC-3', reverse 5'-TCC ATG CTG TTT CTT ACT CTC-3'); NANOG (forward 5'-GAA ATA CCT CAG CCT CCA GC-3', reverse 5'-GCG TCA CAC CAT TGC TAT TC-3'); PAI-1 (forward 5'-CAG AAA GTG AAG ATC GAG GTG AA C-3', reverse 5'-GGA AGG GTC TGT CCA TGA TGA A-3'); ALK-5 (forward 5'-CTG GGA AAT TGC TCG ACG ATG-3', reverse 5'-ACT CTC AAG GCT TCA CAG CTC-3'); TGF-β1 (forward 5'-GCC CTG GAC ACC AAC TAT TG-3', reverse 5'-CGT GTC CAG GCT CCA AAT G-3'); TGF-β2 (forward 5'-AAG CTT ACA CTG TCC CTG CTG C-3', reverse 5'-TGT GGA GGT GCC ATC AAT ACC T-3'); TGF-β3 (forward 5'-TCA GCC TCT CTC TGT CCA CTT-3', reverse 5'-CAT CAC CGT TGG CTC AGG G-3'); SMAD2 (forward 5'-GCA CTT GCT CTG AAA TTT GGG C-3', reverse 5'-GAC GAC CAT CAA GAG ACC TGG-3'); SMAD3 (forward 5'-GCC TGT GCT GGA ACA TCA TC-3', reverse 5'-TTG CCC TCA TGT GTG CTC TT-3'); SMAD4 (forward 5'-GGT TCC TTC AAG CTG CCC TA-3', reverse 5'-ATG TGC AAC CTT GCT CTC TCA-3').

Immunoblot analysis

Whole cell lysates were prepared using radio-immunoprecipitation assay (RIPA) lysis buffer (pH 7.8) containing 25 mM Tris-HCl, 120 mM NaCl, 5 mM EDTA and 0.5% NP-40 supplemented with 2 µg/mL aprotinin, 10 µg/mL leupeptin, 100 µg/mL phenylmethylsulfonyl fluoride (Sigma Aldrich), 200 mM sodium orthovanadate, 0.5 M NaF, protease inhibitor cocktail sets III and IV (Sigma Aldrich) and phosphatase inhibitor cocktails 2 and 3 (Sigma Aldrich). Protein levels were determined using the Thermo Scientific Pierce bicinchoninic acid assay (BCA) protein assay kit. Equal

protein concentrations (25 µg) were loaded and electrophoresis was performed on SDS-PAGE (8-10%) under reducing conditions (5**%** v/v, final concentration 2-mercaptoethanol). After electrophoresis, the separated proteins were transferred to nitrocellulose (Bio-Rad) membranes. Next, the membranes were blocked to avoid nonspecific binding of antibodies in TBST containing 5% skim milk or 5% bovine serum albumin (BSA) and incubated overnight at 4°C with primary antibodies. Primary antibodies were as follows: rabbit anti-phospho-AKT (#9275S, CST and #9271S, CST), rabbit anti-AKT (#9272S, CST), rabbit anti-phospho-ERK (#9101S, CST), rabbit anti-ERK (#9102S, CST), rabbit anti-phospho-SMAD2 (#3108S, CST), rabbit anti-SMAD2 (#3122S, CST), rabbit anti-phospho-SMAD3 (#EP823, ab52903), rabbit anti-SMAD3 (#39513, CST), rabbit anti-SMAD4 (#9515S, CST), rabbit anti-phospho-c-MET (Tyr 1234/1235) (D26) (#3077, CST), mouse anti-c-MET (#3148S, CST) or goat anti-actin (#sc 1616, Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were exposed to HRP-conjugated secondary species-specific antibodies (Santa Cruz Biotechnology).

Spherogenicity assay

Spherogenicity assays were performed by seeding 300 cells/well in NBM using 96-well plates and allowing them to form spheres for a period of at least 15 days. Spherogenicity was assessed by counting the number of spheres. Data are expressed as mean ± SD.

Flow cytometry

The cells were dissociated with Accutase™ (Chemie Brunschwig, Basel, Switzerland). For cell cycle analysis 10^6 cells/condition were fixed and permeabilized with cold 70% ethanol. After two PBS washes, the cells were treated with RNase A (Gibco, Grand Island, NY) for 30 min at 4°C to remove RNA and ensure only DNA is stained with propidium iodide (PI) (Sigma-Aldrich). For cell death analysis, 10^5 cells were re-suspended in Annexin buffer [10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH= 7.4] and incubated with Annexin-V-FITC (BD Biosciences, Franklin Lakes, NJ) and PI (Sigma-Aldrich) containing 0.1% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature in the dark. Cells were re-suspended in flow cytometry buffer [PBS, 0.5% BSA, 0.02% NaN3, 1 mM EDTA] and cell death and

cell cycle phases were analyzed by flow cytometry in a BD FACSVerseTM flow cytometer (Becton Dickinson AG, Allschwil, Switzerland). Data were analyzed using FlowJo Software, version 10.0.8 (Ashland, OR).

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of $2x10^6$ cells were collected after 24 h and concentrated using an Amicon Ultra centrifugal filter (3K) (Millipore, Temecula, CA). For quantitative detection of secreted HGF, a HGF ELISA kit (#KAC2211, Invitrogen, Basel, Switzerland) was used according to the manufacturer's protocol.

RNA interference

To silence the expression of *SMAD2, SMAD3*, *SMAD4* and *ALK-5* GIC were transiently transfected by electroporation (Neon transfection system, Invitrogen) using siRNA pools (100 nM final concentration), containing four selected siRNA duplexes, each with a modification pattern that eliminates off-target effects (ON-TARGET plus human SMAD2 siRNA- SMART pool L-003561-00, ON-TARGET plus human SMAD3 siRNA- SMART pool L-020067-00-0020, ON-TARGET plus human SMAD4 siRNA- SMART pool L-003902-00 and ON-TARGET plus human ALK-5 siRNA- SMART pool L-003929-00) (Dharmacon, Lafayette, CO). Non-targeting siRNA pool was used as a negative control. Lentiviral pGIPZ vectors encoding *c-MET*-specific (Oligo ID V3LHS_642486) or non-silencing control shRNA lentivirus (Oligo ID RHS4346) were purchased from Thermo Scientific (Waltham, MA). Lentiviral particles were produced in HEK 293T cells using pGIPZ shRNAmir lentiviral vector, pCMV-dR8.91 packaging and pMD2.G envelope plasmids. Glioma cells were transduced with lentiviral particles and stably transduced clones were isolated with 4 µg/ml puromycin and subjected to analyses after 1-5 passage post-selection. Efficiency of gene silencing was assessed by RT-PCR and immunoblot.

Immunocytochemistry (ICC) and immunohistochemistry (IHC)

Single or double antigen labeling of formalin-fixed 4-µm-thick sections by ICC or IHC included the following steps: deparaffinization, rehydration of sections and following heat-induced antigen retrieval by boiling in EDTA buffer (1 mM EDTA, 0.05% Tween 20, pH 8.0) for 15 min in a steamer, treatment with 1% H_2O_2 for 15 min, blocking in

SuperBlock solution (#AAA125, ScyTek Laboratories, Logan, UT) for 30 min at room temperature in a humid chamber and primary antibody incubation. The following primary antibodies were used: rabbit anti-phospho-c-MET (Tyr1234/1235) polyclonal antibody (# AF2480, R&D Systems), isotype rabbit IgG control (#ab27472, abcam, Cambridge, UK), mouse monoclonal anti-TGF-β2 antibody (# ab36495, abcam) or mouse IgG1 monoclonal (NCG01) - isotype control (# ab81032, abcam). Incubation with these antibodies was followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (#sc-2030, Santa Cruz) or the VECTASTAIN ABC-alkaline phosphatase (AP) detection system (containing biotinylated anti-mouse IgG) (#AK-5002, Vector Labratories, Burlingame, CA). The HRP green precipitate is ideal for double staining given its good contrast to red chromogenic substrates used with AP detection systems. For color development Permanent HRP Green (#ZUC070-100, Zytomed System, Berlin, Germany) and VectorRed (#SK-5100, Vector Labratories) substrate kits were used. In double stain procedures, VectorRed was used as the last chromogen. Specificity verification and titration of primary and secondary antibodies were carried out first by ICC. To perform reliability testing for the anti-TGF-β2 antibody, TGF-β2-negative ZH-161 and ZH-305 cells with moderate levels of TGF-β2 expression were selected. Similarly, to test the anti-phospho-c-MET antibody, c-MET/p-c-MET-negative T-269 and c-MET/p-c-MET-positive ZH-161 cells were used. The 3D-cultured GIC spheres were embedded into paraffin blocks without trypsinization according to a slightly modified protocol (Pinto, 2011). Embedded cells were sectioned and subsequently subjected to standard staining procedures as specified above. The evaluation of antigen expression in human tumors *in vivo* was performed on tumor nodules, which were demarcated from the normal brain tissue based on histomorphological features of glioblastoma including the presence of microvascular proliferation, prominence of glomeruloid vessels, nuclear atypia, high mitotic activity confirmed by KI-67 staining and necrosis. The immunohistochemically stained sections were viewed by a Nikon Eclipse 80i (Nikon Corporation, Tokyo, Japan) microscope with a Nikon CFI Plan Apo Lambda 40X (Nikon Corporation) bright field objective, Olympus UC30 (Olympus K.K., Tokyo, Japan) camera and processed by cellSens Entry 1.12 by Olympus Corporation (Olympus K.K.) software. To compare the proportion of each antigen to the other in the same tumor specimen, the quantification of each chromogen was performed separately within three randomly selected different

microscopic fields of each specimen using Image J (version 1.32j) software (National Institutes of Health, http://rsb.info.nih.gov/ij/). To separate different chromogens a color deconvolution module was used. TGF-β2 and p-c-MET immunoreactivities were determined by measuring the integrated density (the sum of the pixel values) per field (tumor region). The results per tumor region were then pooled and averaged for each patient and for each chromogen separately. A cutoff of 5% stained tumor cells was used to allocate a sample to the TGF-β2 and p-c-MET high or low groups. The staining procedure, image acquisition, threshold settings were identical for the entire set of patient samples. Clinical information on the cohort of 58 newly diagnosed and 8 recurrent glioblastoma patients used in this study has been published (Frei *et al.*, 2015).

Statistical analysis

All *in vitro* experiments reported here were performed in biological and technical replicates. Results of representative experiments are shown. Quantitative data were expressed as the mean and SD of triplicate determinations. The statistical analyses were performed by student *t*-test and one-way ANOVA with Tukey's multiple comparison tests wherever applicable (GraphPad Software, La Jolla, CA). A *p* value below 0.05 was considered significant. The Spearman's rank correlation coefficient was calculated to analyze the statistical association between the mean of TGF-β2 and p-c-MET levels (integrated density values) *in vivo*.

5. Aims of the thesis

Therapeutic resistance in glioblastoma has been recently attributed to the complex network of interactions between oncogenic signaling cascades. Approaches targeting specific pathways lead to compensatory mechanisms initiated by other functionally redundant signaling, thus rendering glioblastoma refractory to therapies. Combinatorial strategies have gained considerable interest in reaching therapeutic efficacy. Among multiple pathways associated with glioblastoma, the HGF/c-MET and TGF-β pathways are considered drivers of resistance to irradiation and maintenance of the treatment-refractory GIC population. Despite the significant role in glioblastoma pathogenesis, their potential interactions remain uncharacterized. Therefore, the aims of this thesis were:

- To characterize patient-derived GIC models for their basal TGF-β and HGF/c-MET pathways activity.
- To evaluate if genetic and pharmacological modulation of the TGF-β signaling pathway influences HGF/c-MET signaling activity in c-MET-positive GIC models.
- To identify the molecular mechanisms involved in TGF-β-dependent control of HGF/c-MET signaling by interfering at different levels with the TGF-β pathway.
- To study the control of glioma stem cell properties by TGF-β and HGF/c-MET pathways alone and by their balance in c-MET-positive *versus* negative GIC models, in order to determine the functional relevance of this molecular interaction.
- To corroborate the results *in vivo* using human glioblastoma specimens.

6. Results and Discussion

6.1 Crosstalk of the HGF/c-MET and TGF-β pathways in glioblastoma

A related manuscript has been submitted

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Experimental contributions:

Eleanna Papa performed all the experiments of this work. Emese Szabo and Michael Weller guided all the experiments of this work.

6.1.1 Results

Characterization of HGF/c-MET pathway and TGF-β ligands expression in GIC

We first analyzed c-MET pathway in three different patient-derived GIC models referred to as ZH-161, ZH-305 and T-269. The levels of HGF and c-MET were assessed in all GIC lines (Fig. 12A and B). Consistent with the expression of *HGF* and *c-MET* mRNA (Fig. 12A) the ZH-161 and ZH-305 models exhibited basal level of phosphorylated thus active c-MET (p-c-MET) as demonstrated by immunoblot and immunocytochemistry (Fig. 12B and C). Despite comparable expression levels of *HGF* and *c-MET* mRNA and total c-MET protein amount in ZH-161 and ZH-305, c-MET phosphorylation was significantly higher in ZH-161. The expression of *HGF* and *c-MET* mRNA as well as c-MET protein levels were below detection limit in T-269 (Fig. 12A and B). In line with the absence of c-MET, these cells were negative for p-c-MET by immunoblot and immunocytochemistry (Fig. 12B and C). Overall, these results allowed us to define ZH-161 and ZH-305 as c-MET-positive GIC models and T-269 as a c-MET-negative GIC model.

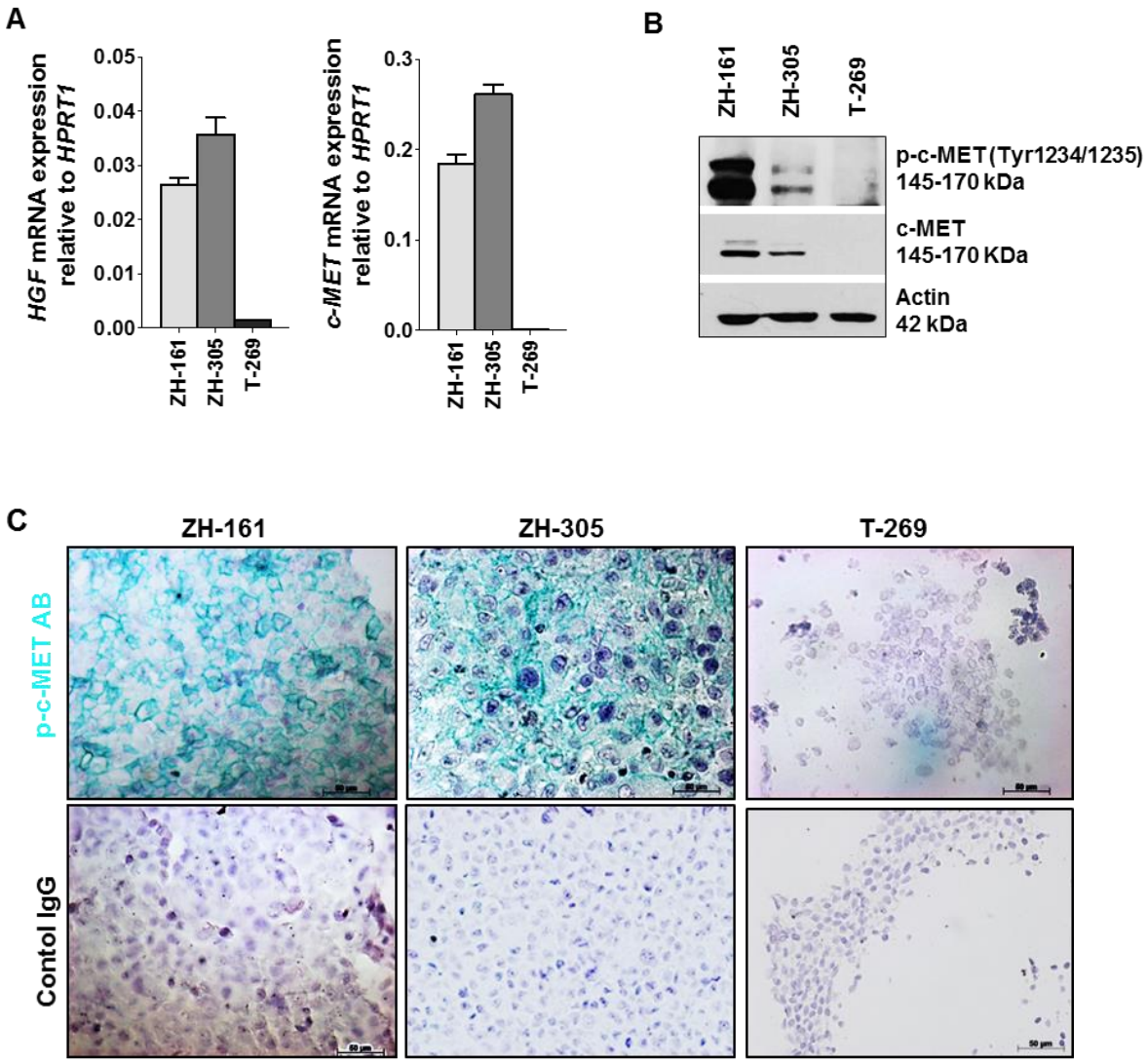


Figure 12. Characterization of the HGF/c-MET pathway in GIC. (A) *HGF* and *c-MET* mRNA levels were quantified by RT-PCR. (B) Equal amount of cellular lysates were assessed for c-MET and p-c-MET levels by immunoblot. Actin was used as loading control. (C) Paraffin-embedded spheres were sectioned and stained for p-c-MET (HRP) using 0.5 µg/ml primary antibody or rabbit isotype control. Nuclei were counter-stained with hematoxylin (blue) (scale bars: 50 µm).

Next, the expression levels of all three *TGF-β* ligands (*TGF-β 1/2/3*) were assessed at mRNA level in the same GIC models. *TGF-β1* and *TGF-β3* expression levels were similar in our GIC panel. *TGF-β2* was predominantly expressed by ZH-305 (Fig. 13A). *TGF-β2* mRNA correlated with TGF-β2 protein levels assessed by immunocytochemistry in ZH-161 and ZH-305 (Fig. 13B).

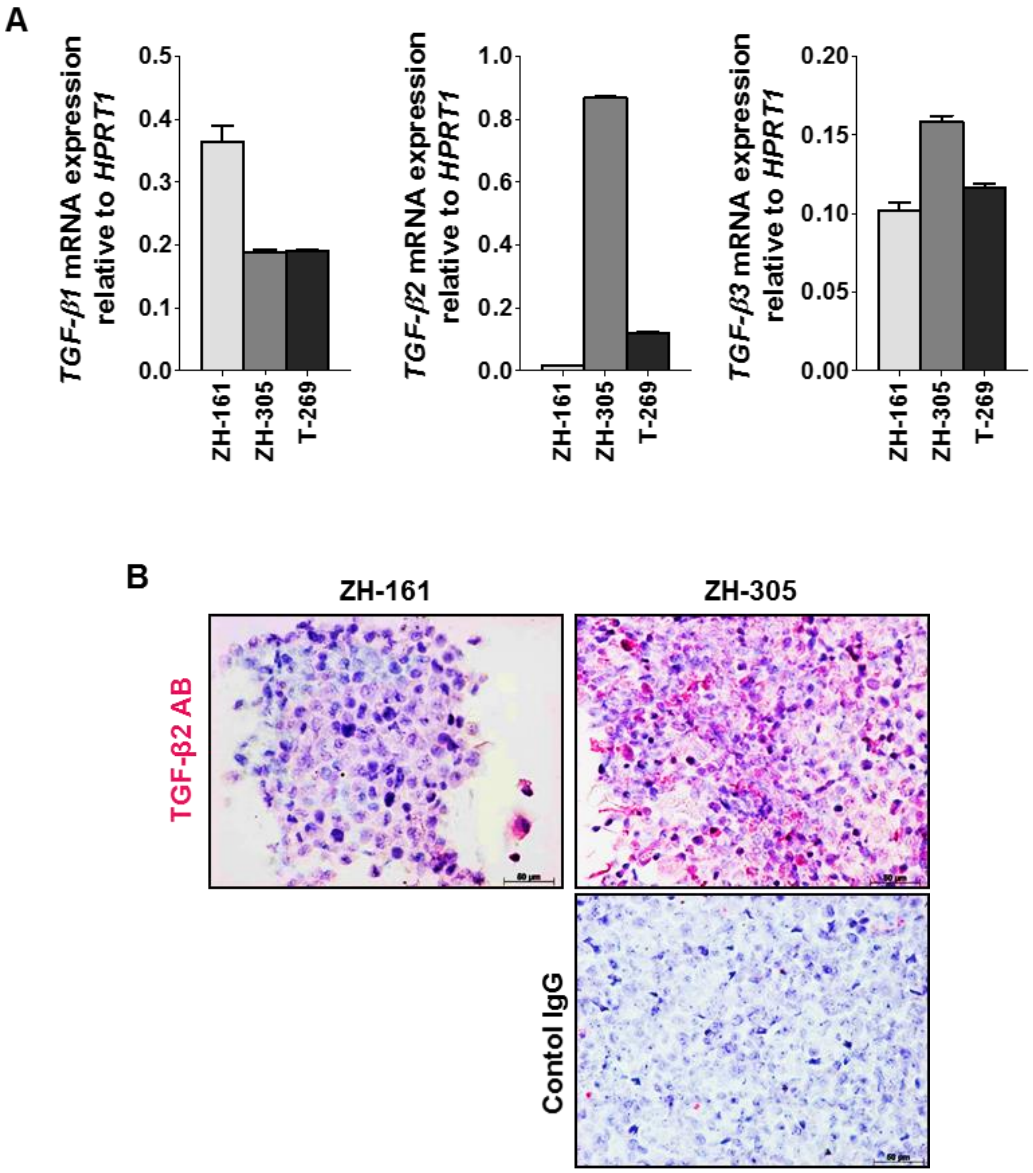


Figure 13. TGF-β ligands in GIC. (A) *TGF-β 1/2/3* mRNA levels were quantified by RT-PCR. (B) Paraffin-embedded spheres were sectioned and stained for TGF-β2 (AP) using 0.5 µg/ml primary antibody mouse isotype control. Nuclei were counter-stained with hematoxylin (blue) (scale bars: 50 µm).

TGF-β suppresses HGF/c-MET pathway activity in glioblastoma

We next investigated the potential effects of TGF-β modulation on c-MET pathway activity using the ZH-161 and ZH-305 models. The exposure of the cells to recombinant TGF-β2 induced activation of TGF-β signaling, as defined by increased p-SMAD2 levels, and resulted in significant reduction of c-MET phosphorilation displayed by immunoblot (Fig. 14A) and immunocytochemistry (Fig. 14B). A minor decrease in total c-MET was observed upon TGF-β2 stimulation, too. The next step was to block TGF-β signaling by the use of a TGF-βRI (ALK-5) inhibitor, SD-208. Although SD-208 did not affect basal p-c-MET levels, it prevented the reduction of p-c-MET when combined with recombinant TGF-β2 (Fig. 14A).

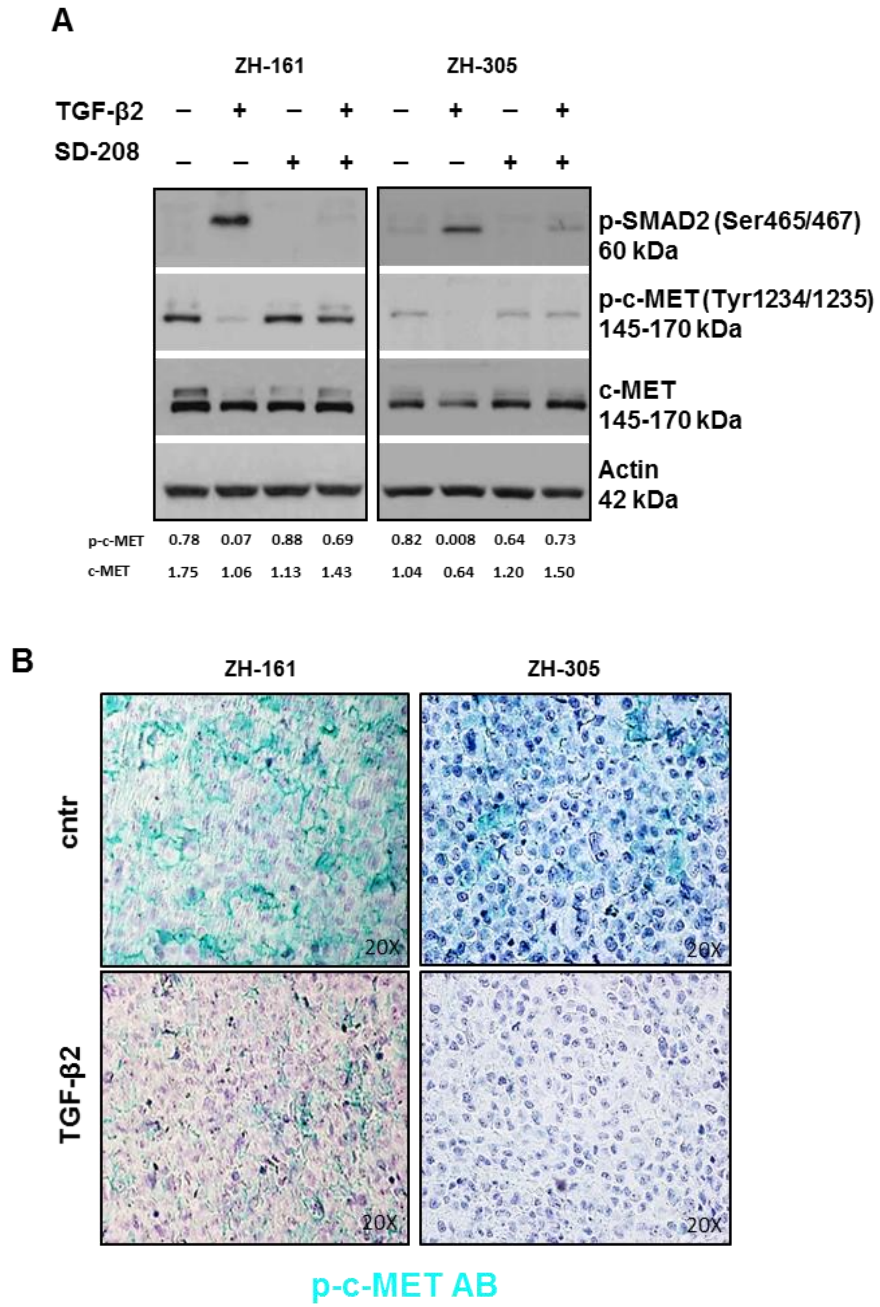


Figure 14. **Control of c-MET activity by TGF-β signaling. (A) GIC were seeded in complete NBM in the presence of SD-208 (1 µM) or TGF-β2 (2 ng/ml) or both for 24 h. DMSO diluted in NBM (1:20'000) served as a control. p-SMAD2, p-c-MET and c-MET protein levels were assessed by immunoblot. 30 µg/lane protein for ZH-161 and 50 µg/lane for ZH-305 were loaded. Actin was used as loading control. Quantification of band intensity by Image J is shown below the immunoblot panels. (B) GIC were seeded in complete NBM in the presence of TGF-β2 (2 ng/ml) for 24 h and subsequently embedded in paraffin. Paraffin-embedded spheres were sectioned and stained for p-c-MET (HRP) using 0.5 µg/ml primary antibody rabbit isotype control. Nuclei were counter-stained with hematoxylin (blue) (scale bars: 50 µm).**

The involvement of TGF-βRI in TGF-β-induced p-c-MET reduction was further confirmed using a second TGF-βRI inhibitor, LY2157299, in ZH-161. Similar to SD-208, LY2157299 did not modulate basal p-c-MET but it prevented the negative effect of exogenous TGF-β2 on c-MET phosphorylation (Fig. 15A). To exclude potential off-target effects of the pharmaceutical inhibitors, we used siRNA oligonucleotides to specifically silence the expression of *ALK-5* in ZH-161. Compared to control transfected cells, we achived a 85% reduction in *ALK-5* expression at mRNA level. Moreover, to prove the role of *ALK-5* mediated signaling, we assessed the expression of *PAI-1*, a recognized downstream target of TGF-β/ALK-5 cascade. We observed that TGF-β2-dependent up-regulation of *PAI-1* was prevented in *ALK-5* siRNA-transfected cells. Consistent with the pharmacologic inhibition of ALK-5, the genetic blockade of this receptor was proven to be effective in countering TGF-β2 negative control of p-c-MET levels (Fig. 15B). Overall, we demonstrated that the TGF-β signaling pathway exerts a negative effect on c-MET signaling in a TGF-βRI-dependent manner.

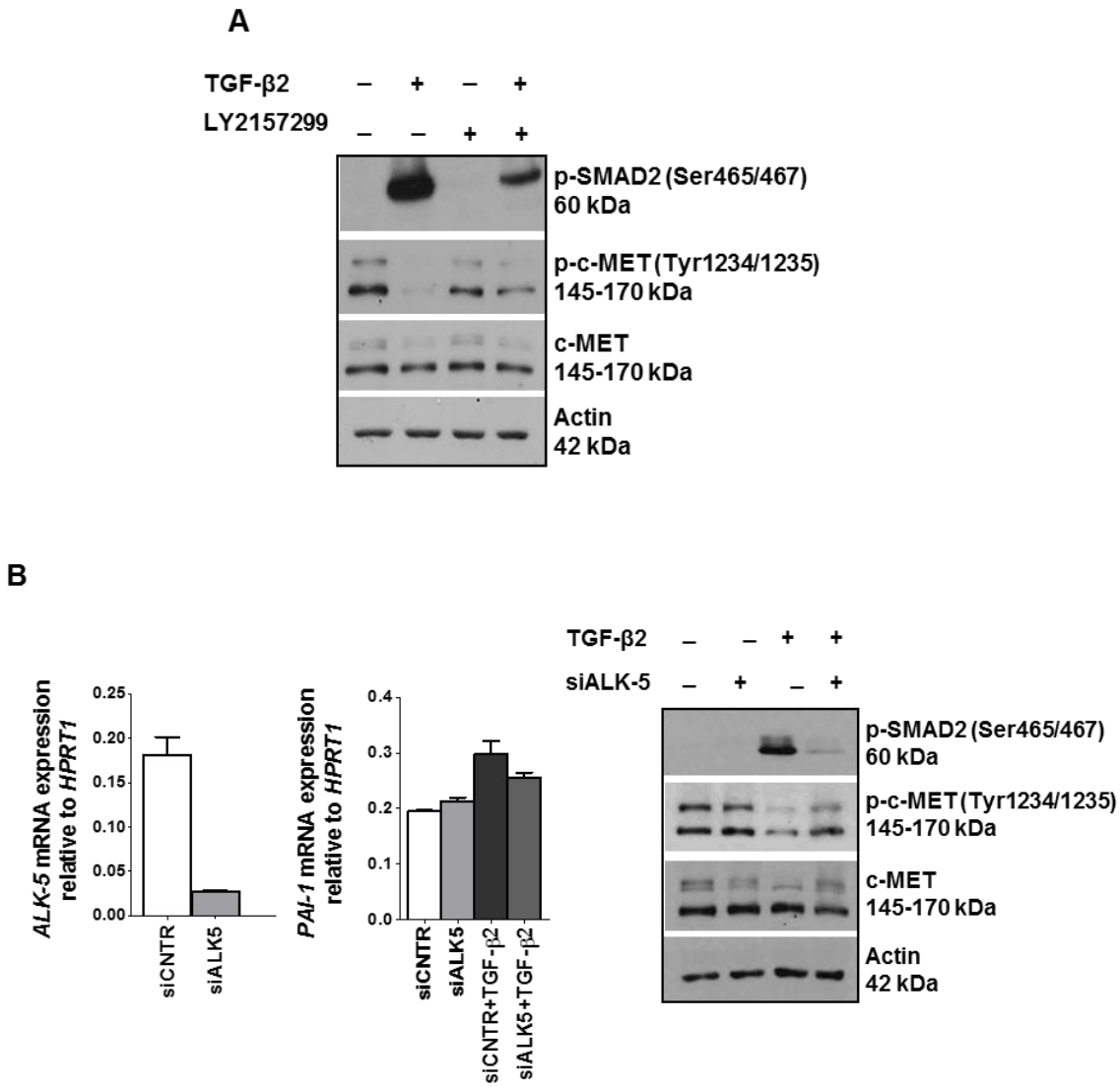
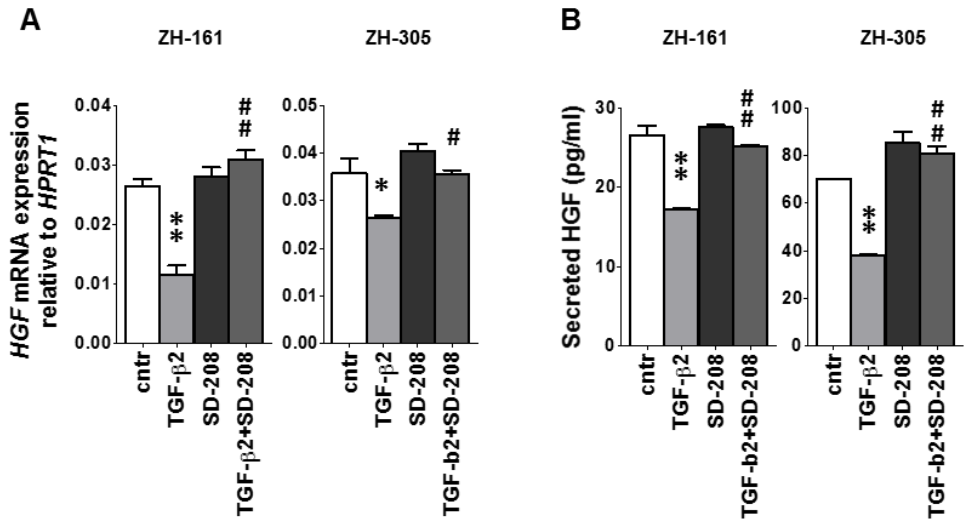


Figure 15. Role of ALK-5 in the control of c-MET activity by TGF-β2. (A) ZH-161 were seeded in complete NBM in the presence of LY2157299 (1 µM) or TGF-β2 (2 ng/ml) or both for 24 h. DMSO diluted in NBM (1:20'000) served as a control. (B) Transient genetic depletion of *ALK-5* was performed by transfecting 100 nM siRNA in ZH-161 using Neon® Transfection System MPK5000. Where indicated, the cells were stimulated 24 h post-transfection with TGF-β2 (2 ng/ml) for 24 h. Control cells were left unstimulated. Silencing efficiency of *ALK-5* (left) and expression levels of TGF-β2 target gene *PAI-1* (middle) were assessed by RT-PCR. Cells under these conditions were assessed for (right) p-SMAD2, p-c-MET and c-MET protein levels by immunoblot. 30 µg/lane protein were loaded. Actin was used as loading control.

TGF-β2 interferes with HGF levels to prevent c-MET phosphorylation

HGF is the only recognized ligand for c-MET receptor and its binding triggers c-MET phosphorylation. This prompted us to determine whether the reduction in p-c-MET upon exposure to TGF-β2 was parallel by a modulation of HGF levels in ZH-161 and ZH-305. We observed that exogenous TGF-β2 significantly reduced *HGF* mRNA expression (Fig. 16A) and protein release detected by RT-PCR and by ELISA assays, respectively (Fig. 16B). The downregulation of HGF expression and secretion upon TGF-β2 was prevented by SD-208 in both cell lines (Fig. 16A and B).



<u>Figure 16.</u> Control of HGF levels by TGF-β signaling. GIC were seeded in complete NBM and stimulated with TGF-β2 (2 ng/ml) for 24 h in the absence or presence of SD-208. (A) Modulation of *HGF* mRNA expression was assessed by RT-PCR. (B) HGF protein release into the supernatant was measured by ELISA (p* < 0.05, ***p* < 0.01, effect of TGF-β2 compared to control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ effect of TGF-β2 and SD-208 co-treatment compared to TGF-β2 alone).**

In contrast, *c-MET* mRNA expression was downregulated by exogenous TGF-β2 in ZH-305 only and it remained stable with co-treatment of TGF-β2 and SD-208 in both cell lines (Fig. 17).

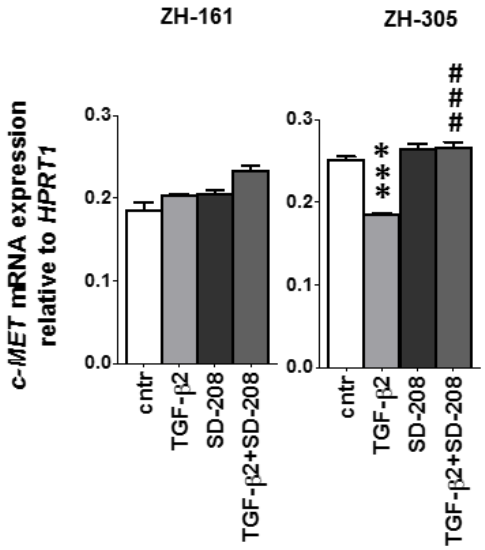


Figure 17. TGF-β2-dependent control of c-MET expression. GIC were seeded in complete NBM in the absence or presence of SD-208 (1 µM) or TGF-β2 (2 ng/ml) or both for 4 h. DMSO diluted in NBM (1:20'000) served as a control. Modulation of *c-MET* mRNA expression in ZH-161 (left) or ZH-305 (right) cells was analyzed by RT-PCR (*$p < 0.001$, effect of TGF-β2 compared to control, $^{###}p < 0.001$ effect of TGF-β2 and SD-208 co-treatment compared to TGF-β2 alone).**

To further prove that the negative control of HGF by TGF-β led to reduction of p-c-MET levels, we stimulated the cells with recombinant HGF and tested whether the p-c-MET levels were rescued by exogenous HGF in the presence of TGF-β2. Indeed, HGF restored p-c-MET levels in TGF-β treated cells (Fig. 18), suggesting loss of *HGF* expression as a mechanism in the negative regulation of c-MET pathway by TGF-β.

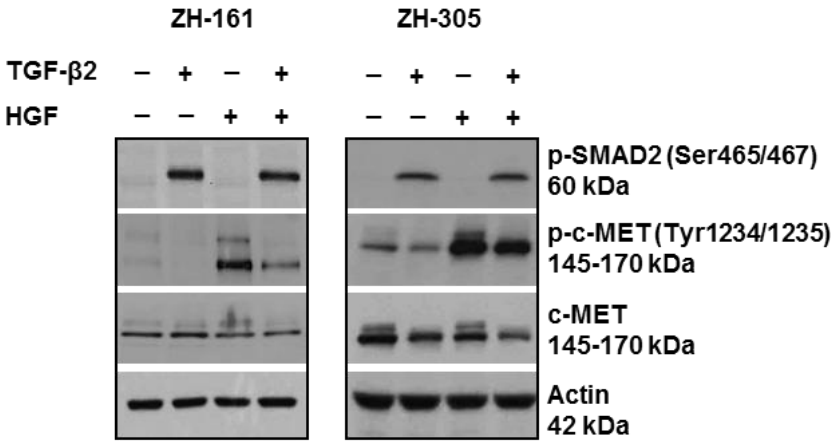


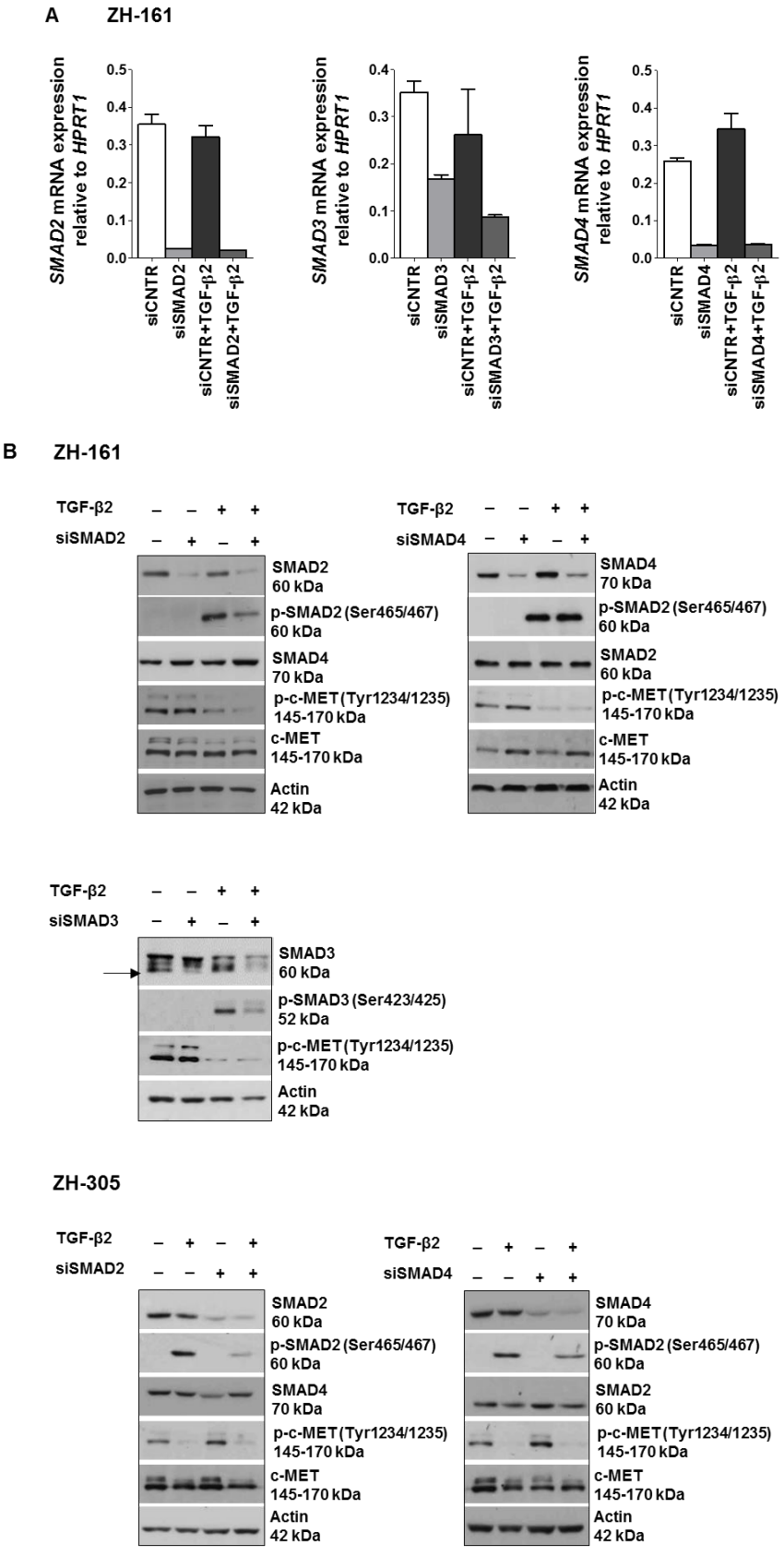
Figure 18. TGF-β2 modulation of HGF-dependent c-MET phosphorylation. GIC were seeded in complete NBM and stimulated with HGF (50 ng/ml) or TGF-β2 (2 ng/ml) or both for 24 h. p-SMAD2, p-c-MET and c-MET protein levels were assessed by immunoblot; 30 µg/lane protein for ZH-161 and 50 µg/lane for ZH-305. Actin was used as loading control.

TGF-β suppression of HGF/c-MET pathway occurs in a SMAD-independent manner

To clarify the molecular events in the TGF-β-dependent suppression of HGF/c-MET pathway activity, we first analyzed the involvement of SMAD proteins, the central effectors of TGF-β canonical signaling. We used siRNA oligonucleotides to specifically silence the expression of *SMAD2*, *SMAD3* or *SMAD4* in ZH-161 and ZH-305. Compared to control transfected cells, SMAD2, SMAD3 and SMAD4 expression levels were significantly reduced at both mRNA (Fig. 19A) and protein (Fig. 19B) levels. In contrast to TGF-βRI inhibition, *SMAD2*, *SMAD3* or *SMAD4* silencing did not counteract the TGF-β2-evoked suppression of c-MET phosphorylation (Fig. 19B).

Silencing of each SMAD did not affect the protein levels of the other SMAD, which confirms the specificity of the siRNA constructs. Moreover, by measuring *PAI-1* expression, we further proved the efficiency of SMAD silencing in preventing TGF-β downstream target activation. We indeed observed that exogenous TGF-β2-dependent up-regulation of *PAI-1* was lost in *SMAD* knockdown cells (Fig. 19C).

These findings allowed us to exclude a role for SMAD proteins in TGF-β-dependent HGF/c-MET pathway inhibition.



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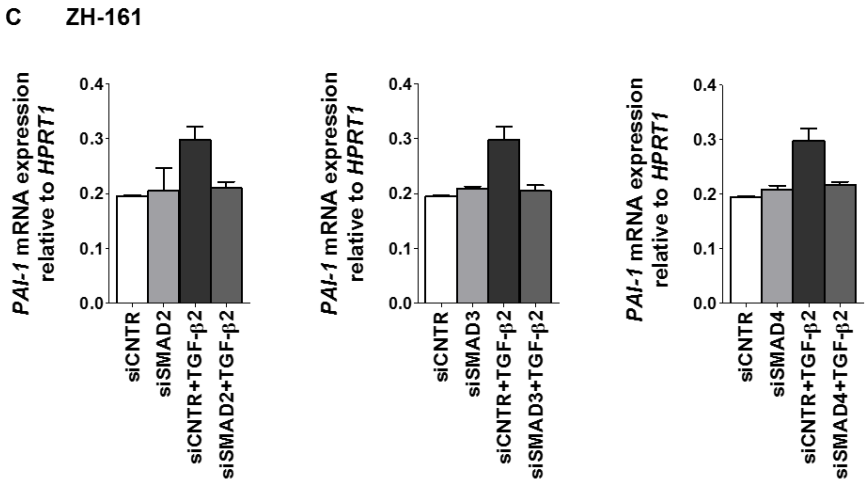


Figure 19. Role of SMAD proteins in the control of the c-MET pathway by TGF-β2. Transient genetic depletion of *SMAD2*, *SMAD3* or *SMAD4* was performed by transfecting 100 nM siRNA in ZH-161 or ZH-305 using Neon® Transfection System MPK5000. Where indicated, the cells were stimulated 2 h post-transfection with TGF-β2 (2 ng/ml). 24 h later, the silencing efficiency of *SMAD2*, *SMAD3*, *SMAD4* was assessed by (A) RT-PCR and (B) by immunoblotting (30 µg/lane protein were loaded). Actin was used as loading control. (C) *PAI-1* gene expression was assessed by RT-PCR. Unstimulated cells were used as references.

TGF-β suppresses HGF/c-MET pathway activity in glioblastoma via MAPK/ERK and AKT non-canonical signaling

In addition to SMAD proteins, TGF-β superfamily members transmit signals via SMAD-independent pathways including various branches of MAPK/ERK and PI3K/AKT cascades. Thus, we assessed whether these pathways might mediate TGF-β-dependent inhibition of HGF/c-MET signaling. We first analyzed the effect of exogenous TGF-β2 on MAPK/ERK cascade activation by measuring the phosphorylated levels of the ERK 1/2 terminal kinase. Exposure to TGF-β2 induced ERK 1/2 phosphorylation in both ZH-161 and ZH-305. The use of U0126, a highly selective inhibitor that blocks the activation of ERK 1/2, interfered with both basal and TGF-β2-induced ERK 1/2 phosphorylation in ZH-161 and ZH-305 (Fig. 20A). We next explored the involvement of ERK 1/2 in TGF-β-dependent modulation of HGF/c-MET pathway. U0126 alone altered neither c-MET phosphorylation (Fig. 20A) nor *HGF* mRNA levels (Fig. 20B), but it counteracted the inhibitory effect of exogenous TGF-β2 on HGF/c-MET pathway in both cell lines. Moreover, U0126

prevented the downregulation of *c-MET* mRNA expression by TGF-β2 in ZH-305 (Fig. 20C).

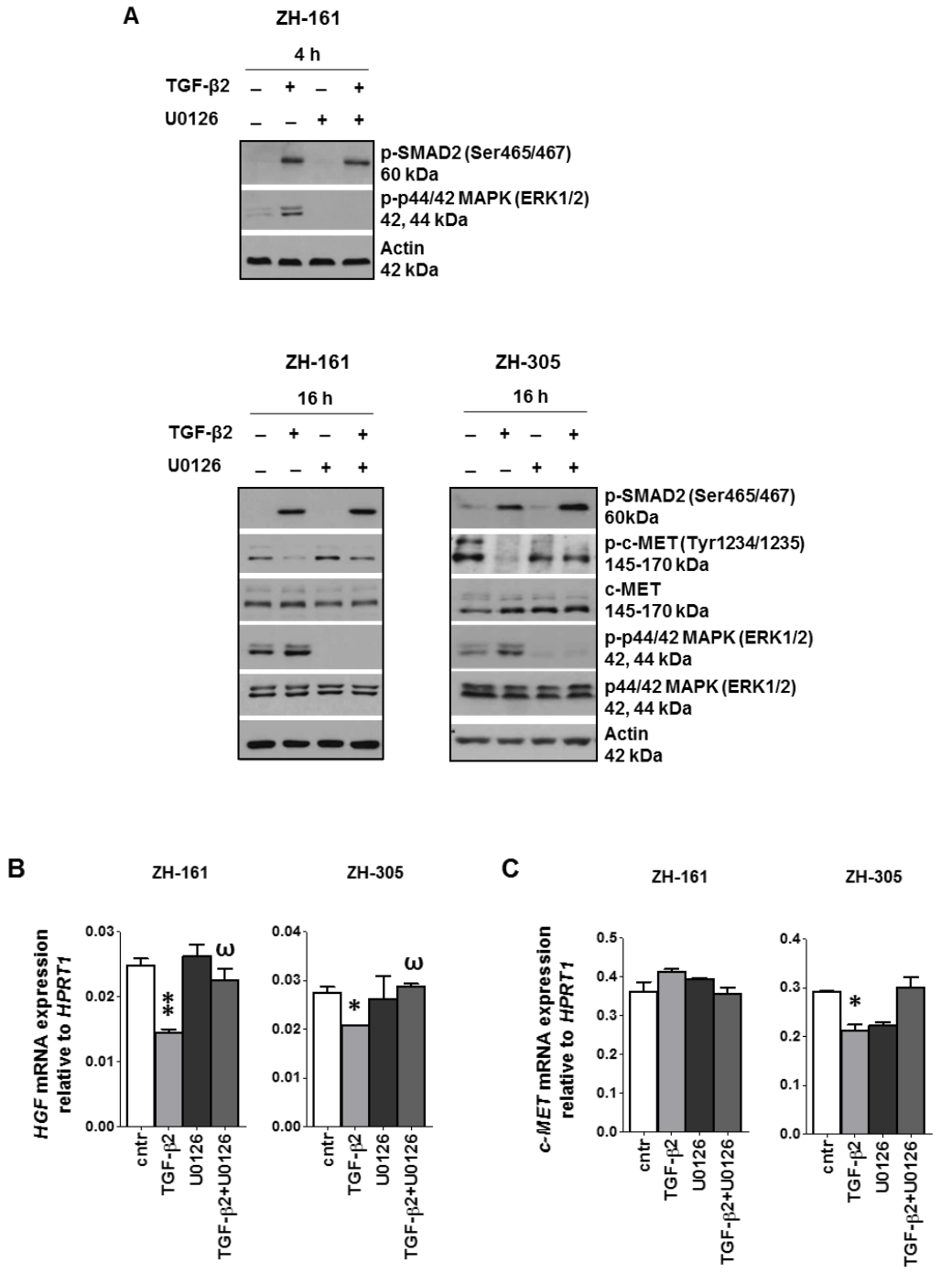


Figure 20. Analysis of MAPK/ERK signaling in the control by TGF-β2 of c-MET activity. GIC were treated with U0126 (10 μM) or TGF-β2 (2 ng/ml) alone or combination and assessed for: modulation of (A, upper panel) ERK phosphorylation (4 h) and (A, lower panels) c-MET activation (16 h) by immunoblot and of (B) *HGF* or (C) *c-MET* mRNA by RT-PCR (4 h) (p* < 0.05, ***p* < 0.01, effect of TGF-β2 compared to control, ᵠp < 0.05, effect of TGF-β2 and U0126 co-treatment compared to TGF-β2 alone).**

The involvement of PI3K/AKT signaling in TGF-β-dependent control of c-MET pathway was also adressed. Therefore, similar analyses as described above were carried out using AZD5363, as a tool to inhibit the phosphorylation of AKT substrate. The increase of p-AKT (Ser473, Thr308) in both ZH-161 and ZH-305 reflects the mode of action of this inhibitor (Davies *et al.*, 2012) (Fig. 21A). Although exposure to TGF-β2 did not modulate p-AKT (Thr308; Ser473) and AZD5363 alone did not modify *HGF* expression, the inhibition of p-AKT by AZD5363 partially rescued both p-c-MET and *HGF* levels in TGF-β2-treated cells (Fig. 21A and B). Further, the downregulation of *c-MET* mRNA expression by TGF-β2 was blocked by AZD5363 in ZH-305 (Fig. 21C).

Overall, these results suggest a role for MAPK/ERK and AKT in the mechanism of HGF/c-MET pathway activation upon exogenous TGF-β in c-MET positive GIC models.

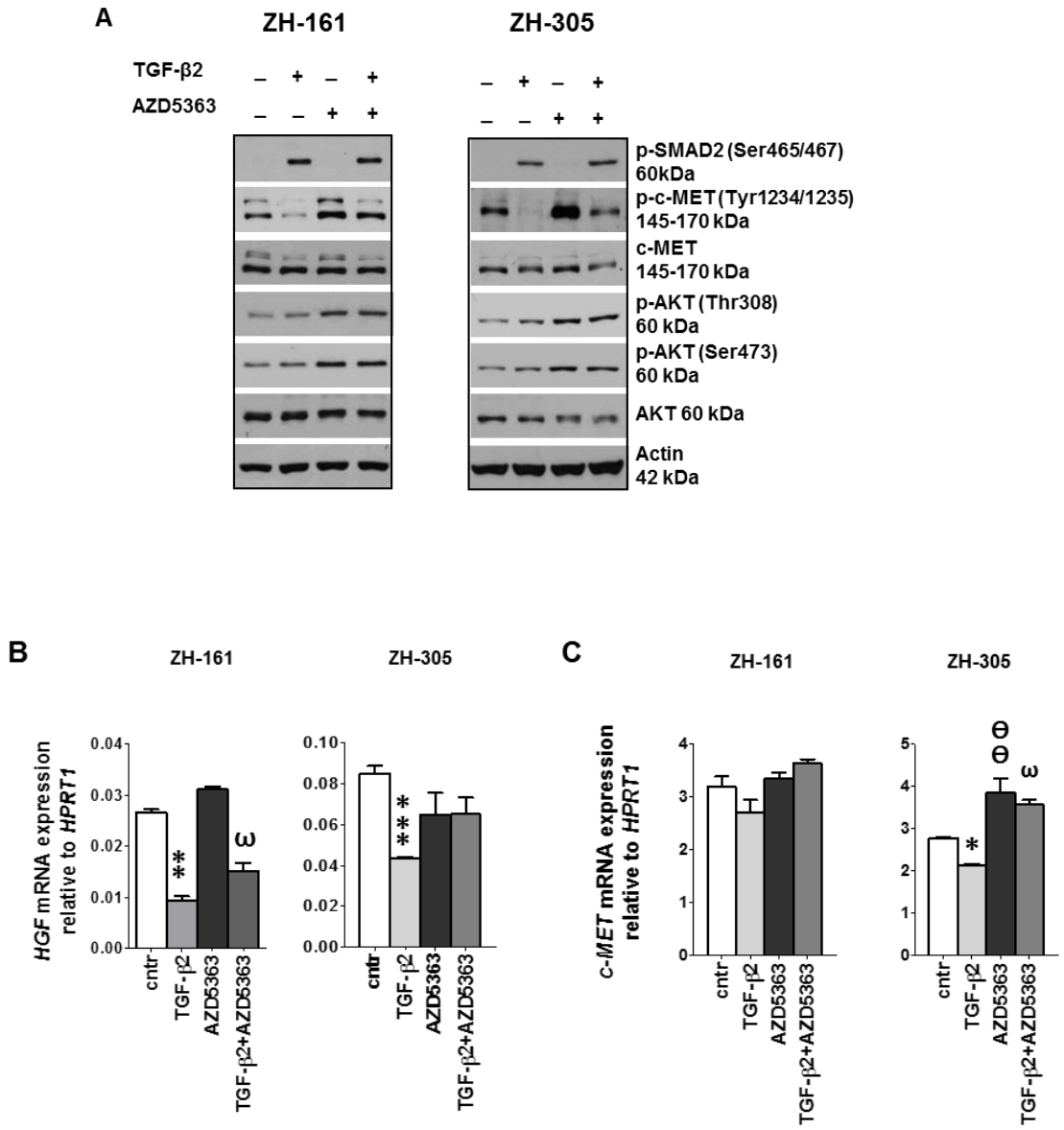


Figure 21. Analysis of the effects of AKT signaling for the control of TGF-β2-dependent modulation of c-MET activity. GIC were treated with AZD5363 (3 μM) or TGF-β2 (2 ng/ml) alone or combination and assessed for: (A) modulation of c-MET activation (16 h) by immunoblot and (B) *HGF* or (C) *c-MET* mRNA by RT-PCR (4 h) upon the treatments. (p* < 0.05, ***p* < 0.01, ****p* < 0.001 effect of TGF-β2 relative to control; $^{\omega}p < 0.05$, effect of TGF-β2 and AZD5363 co-treatment relative to TGF-β2; $^{\Theta\Theta}p < 0.01$, effect of AZD5363 relative to control).**

Context-dependent role of HGF/c-MET and TGF-β signaling in stemness

We next sought to investigate the functional relevance of the interactions observed between the TGF-β and c-MET pathways. It has been shown that aberrant activation of these two cascades contributes to the maintenance of stem-like features of GIC. This function is exerted by their control of the expression of stem-like factors, essential for pluripotency, such as OCT-4, SOX-2 and NANOG. Thus, we first evaluated how these two pathways alone and their balance influence the expression of stem-like markers. We stimulated or inhibited TGF-β signaling by exposure of the cells to recombinant TGF-β or SD-208, alone or in combination. In addition, we treated the cells with EMD1214063, an ATP-competitive inhibitor preventing the activation of c-MET. The inhibition of TGF-β signaling by SD-208 did not affect *OCT-4*, *SOX-2* or *NANOG* mRNA expression. TGF-β2 stimulation resulted in an approximately 2-fold decrease of these stem cell markers at mRNA expression levels in c-MET-positive ZH-161 and ZH-305 cells, except of *OCT-4* in ZH-305. This reduction was prevented when GIC were exposed to recombinant TGF-β2 in combination with SD-208. Similar to exogenous TGF-β2, inhibition of c-MET by EMD1214063 resulted in transcriptional repression of the stem-like markers (Fig. 22A and B). In contrast, none of these markers were affected by TGF-β2 or EMD1214063 in c-MET-negative T-269 cells (Fig. 22C).

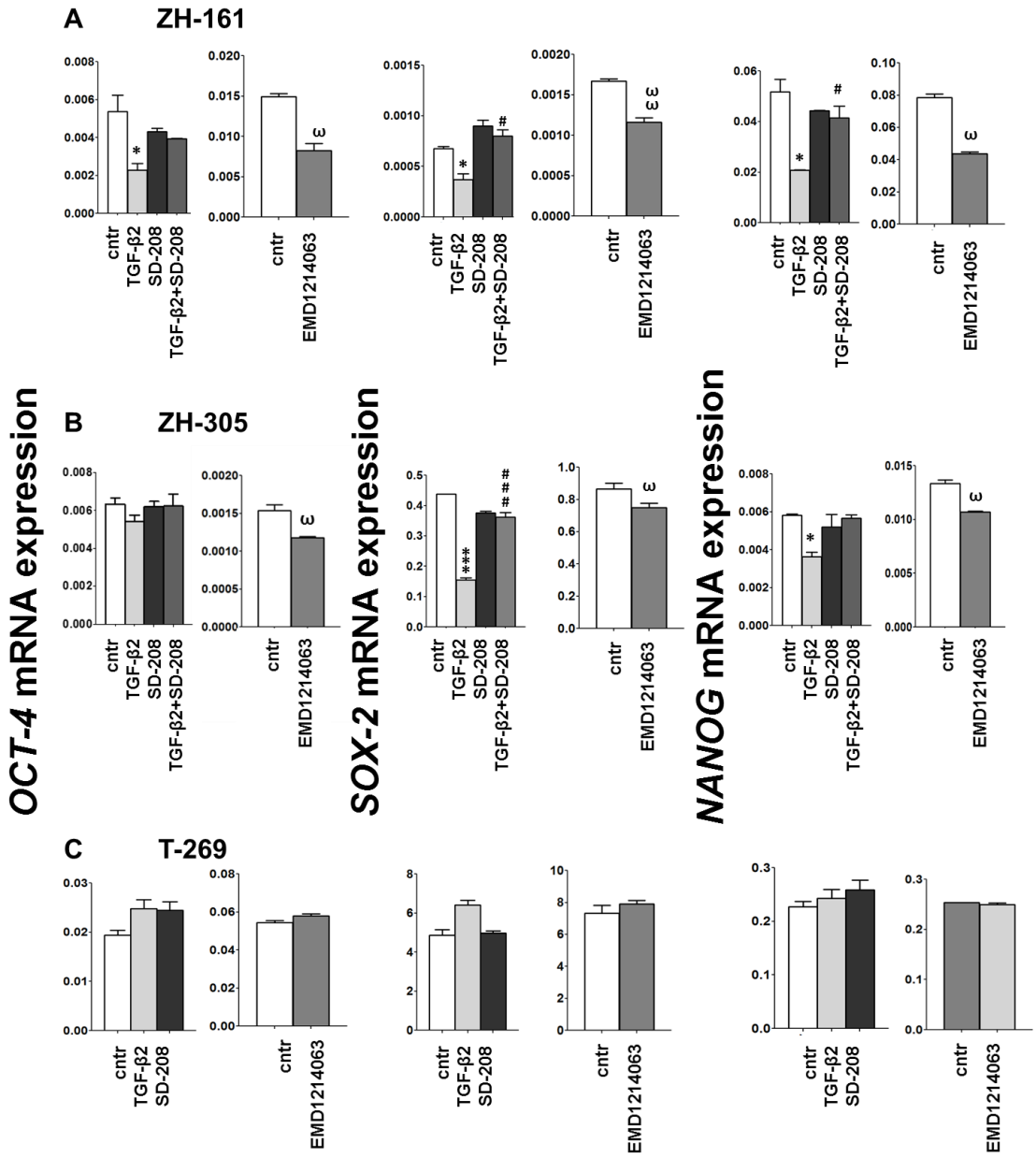
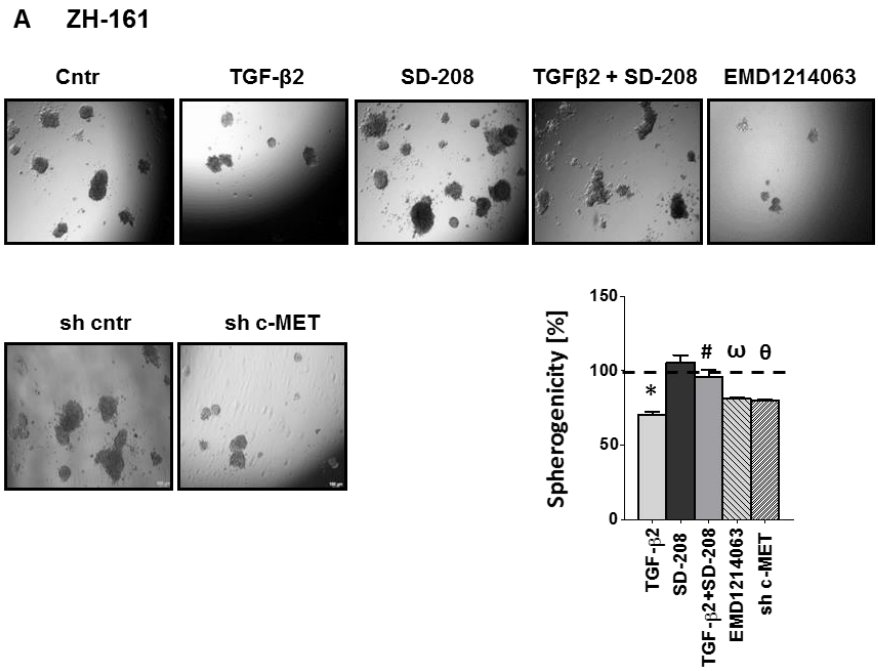
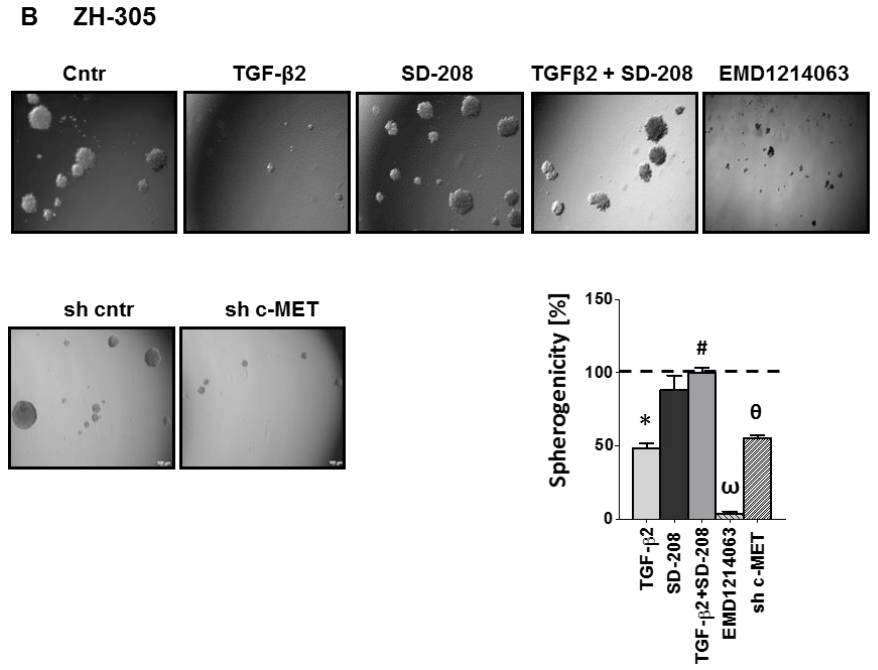


Figure 22. Regulation of stem cell marker expression by TGF-β or c-MET modulation. *OCT-4*, *SOX-2* or *NANOG* gene expression in (A) ZH-161, (B) ZH-305, (C) T-269 upon exposure to TGF-β2 (2 ng/ml), SD-208 (1 µM), or both (4 h) were assessed by RT-PCR (*$p < 0.05$, ***$p < 0.001$, effect of TGF-β2 relative to control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ effect of TGF-β2 and SD-208 relative to TGF-β2). The c-MET inhibitor EMD1214063 (200 nM) was included as a reference ($^{\omega}p < 0.05$, $^{\omega\omega}p < 0.01$).

The expression of stem-like markers was described to correlate with the ability of GIC to grow as neurospheres and to self-renew. Therefore, the second step was to characterize if the reduction of the stem-like marker expression by TGF-β2 as well

as by blockade of c-MET pathway influence GIC spherogenicity. We cultured the GIC models in the presence of TGF-β2 or SD-208, or both, HGF, EMD1214063 or we stably silenced c-MET expression by lentiviral construct (c-MET shRNA) and then analyzed the effect on self-renewal of these cells. Consistent with the downregulation of stem-like marker expression, the exposure to recombinant TGF-β2 of c-MET-positive GIC decreased spherogenicity in a SD-208-sensitive manner. Similarly, lentivirus-mediated c-MET gene silencing or c-MET inhibition with EMD1214063 reduced sphere formation in ZH-161 and ZH-305 GIC models (Fig. 23A and B). Conversely, none of these treatments affected the capability to form spheres in c-MET negative T-269 (Fig. 23C). The efficiency of c-MET silencing was proven by a significant reduction of total and phosphorylated c-MET levels in ZH-161 and ZH-305 models by immunoblot (Fig. 23D).





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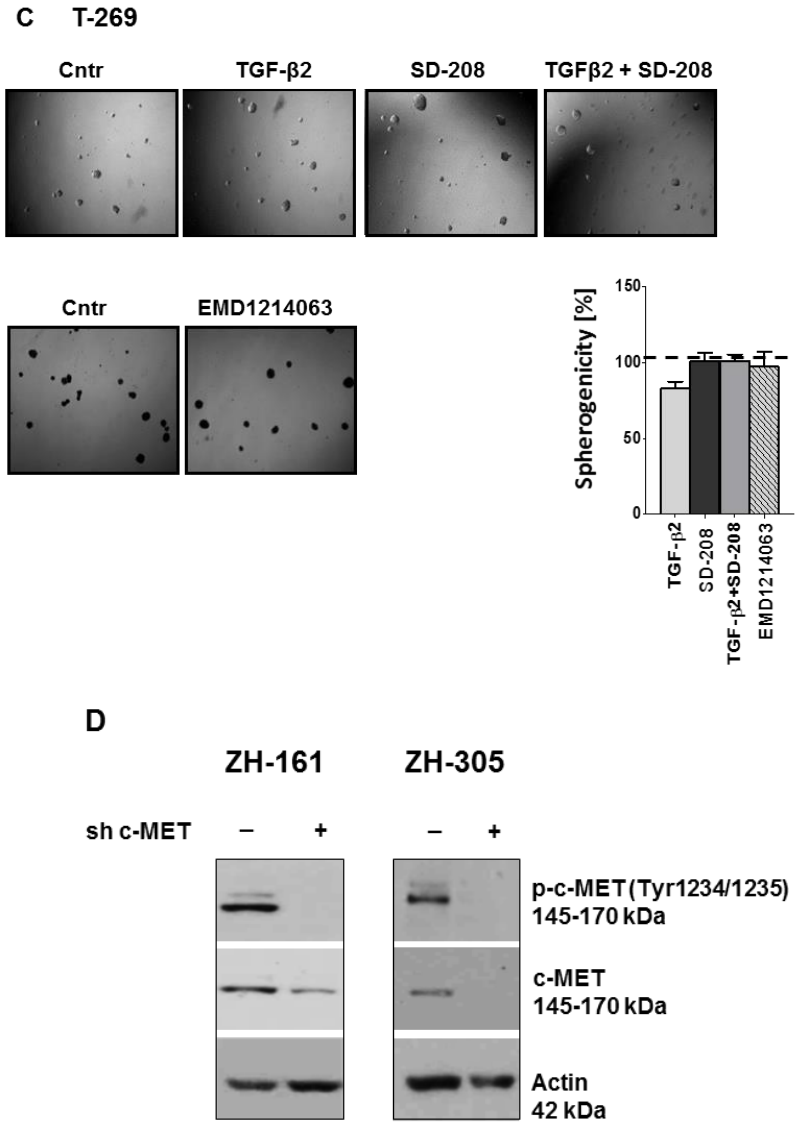
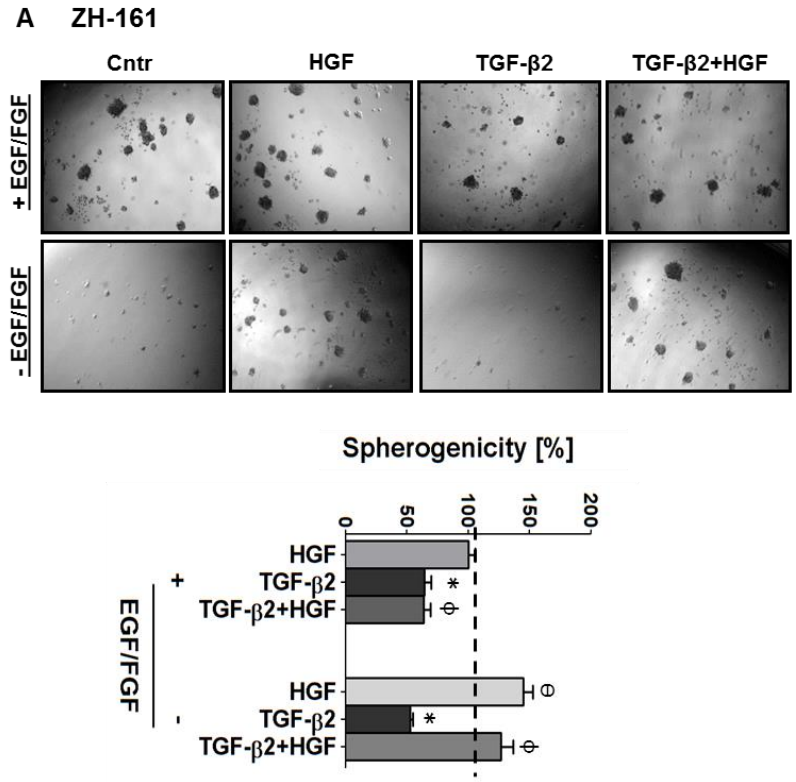
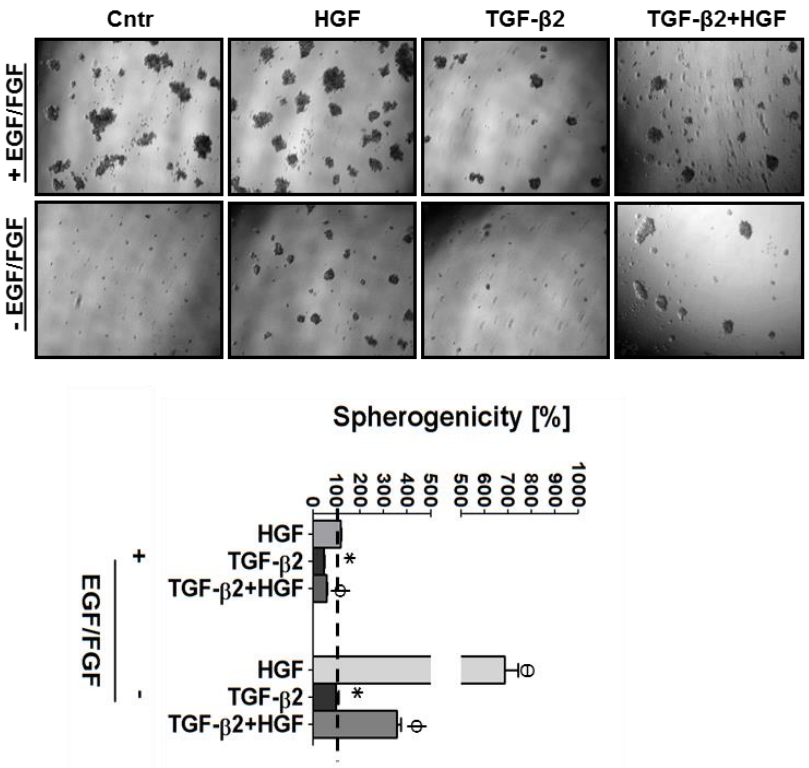


Figure 23. Effect on spherogenicity of TGF-β or c-MET modulation. (A) ZH-161, (B) ZH-305 or (C) T-269 cells were plated at 300 cells/well in NBM in the absence or presence of SD-208 (1 μM) or TGF-β2 (10 ng/ml) or SD-208 combined with TGF-β2 in 96-well plates. ZH-161 or ZH-305 cells were either expressing *c-MET* shRNA or treated with EMD1214063. Spherogenicity was determined by counting spheres. Sphere formation was assessed at day 21 in triplicates (*$p < 0.05$, effects of TGF-β2 compared to control, $^{\#}p < 0.05$, effect of TGF-β2 and SD-208 co-treatment compared to TGF-β2 alone; $^{\omega}p < 0.05$, effect of EMD1214063 compared to control, $^{\theta}p < 0.05$, effect of c-MET shRNA compared to control). (D) Verification of the specificity of the 145 kDa bands and efficacy of *c-MET* gene silencing in c-MET positive GIC by immunoblot for c-MET and p-c-MET. 30μg/lane protein were loaded. Actin was assessed as reference.

Either the activation of TGF-β signaling or the inhibition of the c-MET pathway led to reduction in the stem-like properties of c-MET positive GIC. To evaluate whether the TGF-β2 effect on stemness was due to its negative control of HGF/c-MET, we analyzed the spherogenicity upon TGF-β2 exposures in the presence of recombinant HGF. GIC were cultured in medium lacking EGF/FGF and further stimulated with recombinant HGF. GIC cultured without EGF/FGF showed a tendency to attach to the plastic surface of a culture dish. The exposure to recombinant HGF significantly enhanced the neurosphere forming capability of both ZH-161 and ZH-305 but not of T-269. Additionally, the stimulation with HGF overcame the reduction in sphere formation observed upon TGF-β2 exposure in ZH-161 and ZH-305 (Fig. 24). Overall, our finding suggested that TGF-β negatively regulates the stem-like properties by negatively controlling HGF/c-MET activation in GIC models where this pathway is a master regulator of stemness.





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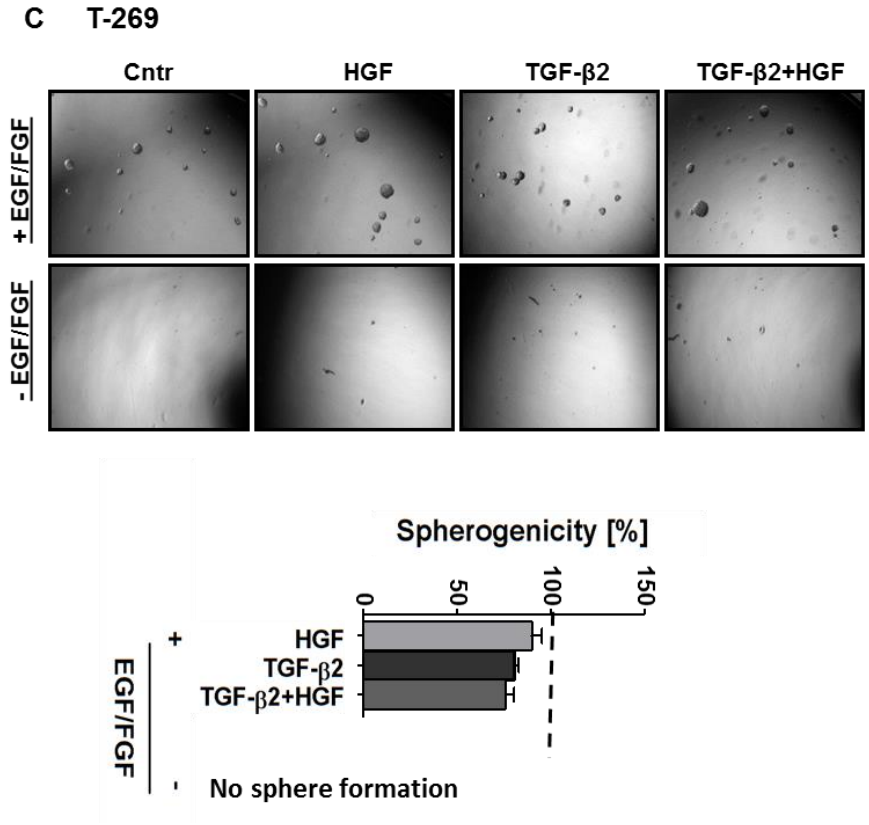
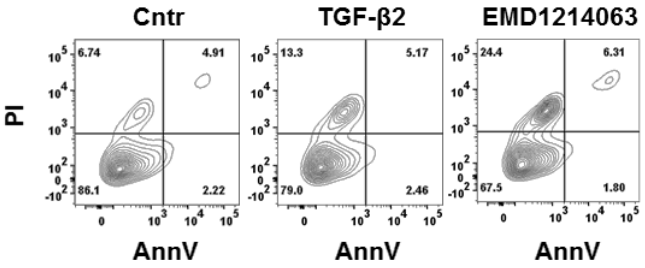


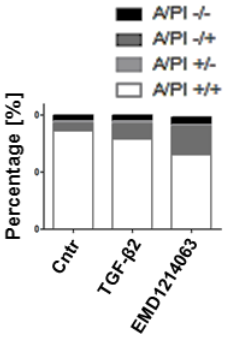
Figure 24. Spherogenicity-promoting role of exogenous HGF. (A) ZH-161, (B) ZH-305 or (C) T-269 cells were seeded at a density of 500 cells/well and grown for 21 days in HGF-containing (50 ng/ml) NBM with (upper panels) or without (lower panels) EGF/FGF. Stimulatory effects of HGF in the absence or presence of TGF-β2 (2 ng/ml) under these conditions were analyzed. Spherogenicity was determined by counting spheres. Sphere formation was assessed at day 21 in triplicates ($^{\theta}p < 0.05$, effect of HGF relative to control; $^{*}p < 0.05$, effect of TGF-β2 relative to control; $^{\Phi} < 0.05$, effect of HGF and TGF-β2 co-treatment relative to TGF-β2).

Moreover, we found that the inhibitory effects of TGF-β2 and EMD1214063 on spherogenicity in ZH-161 and ZH-305 might also involve minor cytotoxicity as demonstrated by changes in viability (Fig. 25A) and cell cycle progression (Fig. 25B) under these conditions.

A

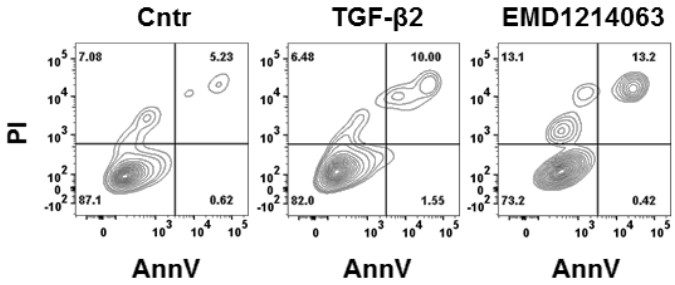
ZH-161

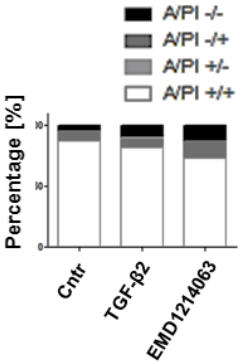




	Cntr	TGF-β2	EMD
A/PI -/-	86.1	79	67.5
A/PI -/+	6.74	13.3	24.4
A/PI +/-	2.22	2.46	1.8
A/PI +/+	4.91	5.17	6.31

ZH-305





	Cntr	TGF-β2	EMD
A/PI -/-	87.1	82	73.2
A/PI -/+	7.08	6.48	13.1
A/PI +/-	0.62	1.55	0.42
A/PI +/+	5.23	10	13.2

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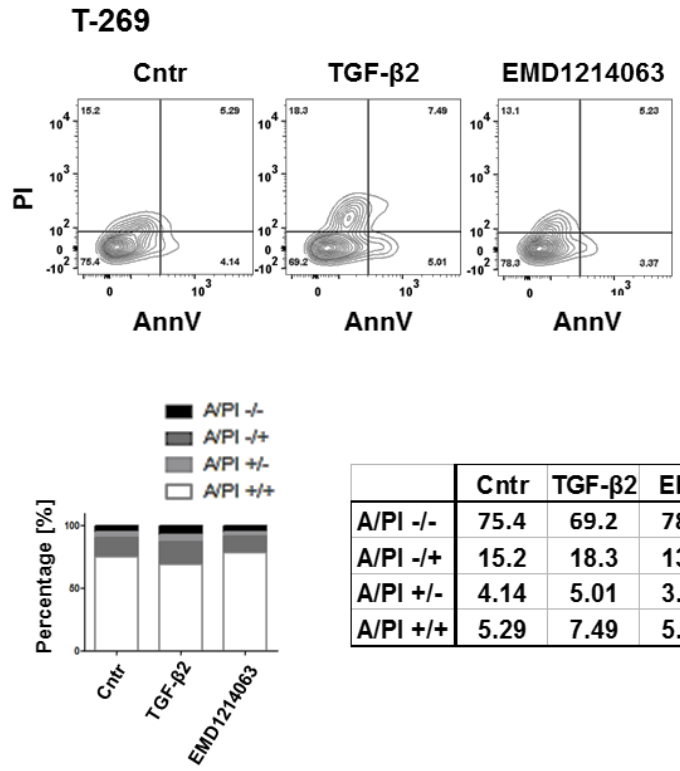
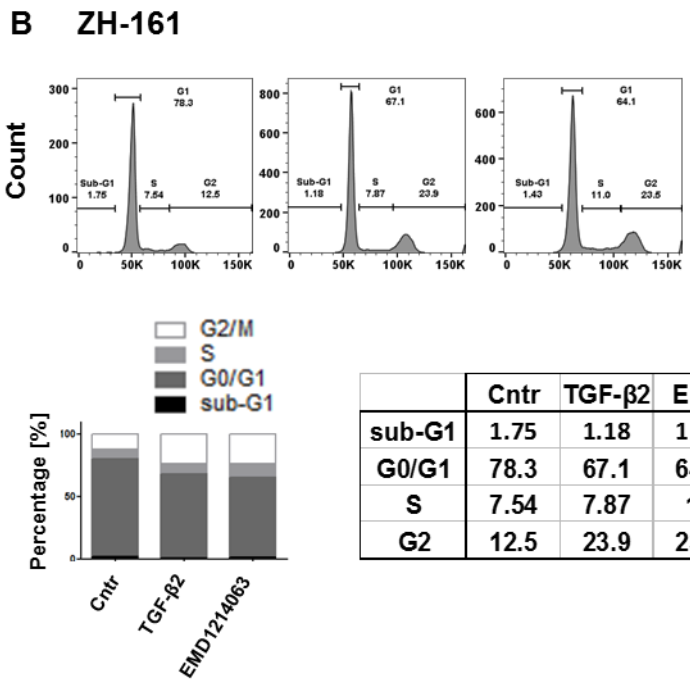
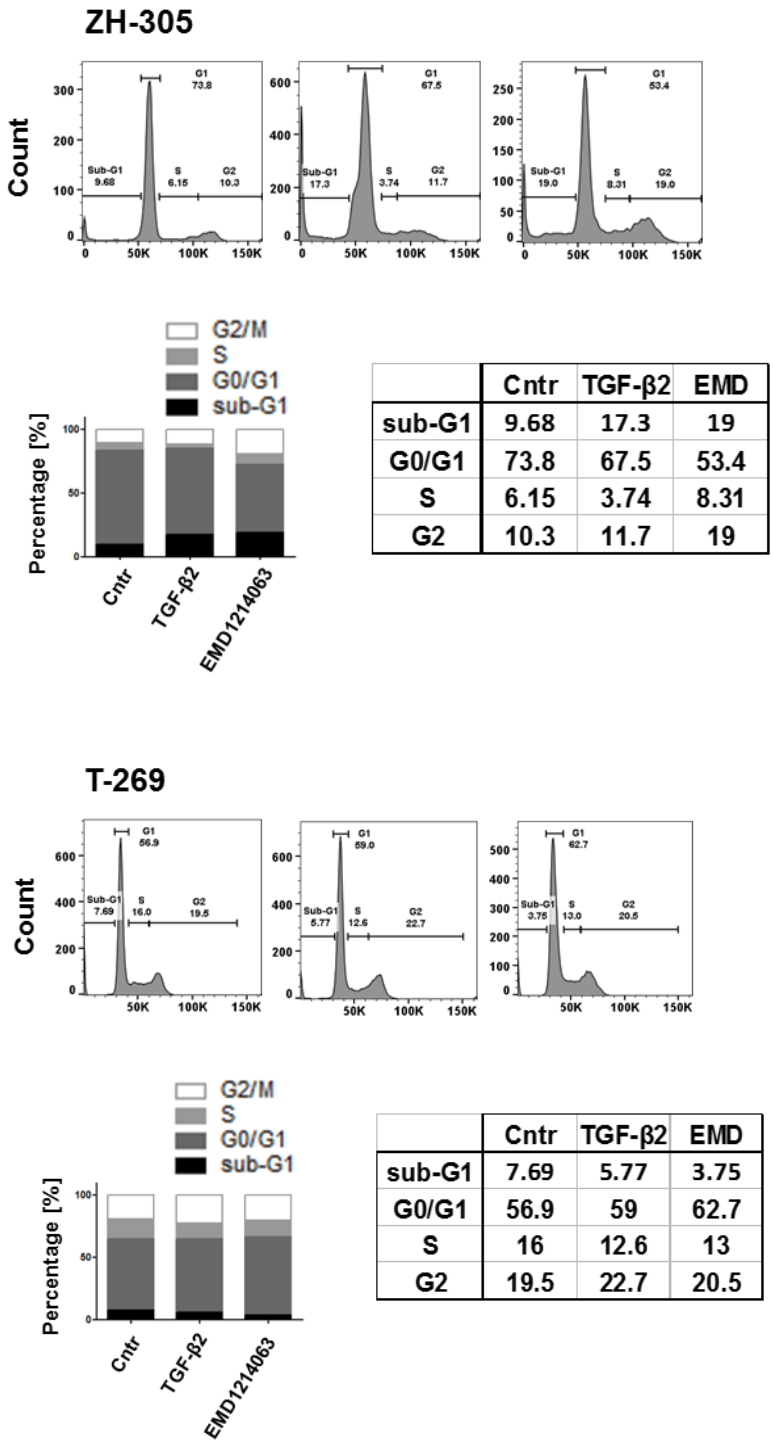


	Cntr	TGF-β2	EMD
A/PI -/-	75.4	69.2	78.3
A/PI -/+	15.2	18.3	13.1
A/PI +/-	4.14	5.01	3.37
A/PI +/+	5.29	7.49	5.23



	Cntr	TGF-β2	EMD
sub-G1	1.75	1.18	1.43
G0/G1	78.3	67.1	64.1
S	7.54	7.87	11
G2	12.5	23.9	23.5

Figure 25 continues on the next page.



	Cntr	TGF-β2	EMD
sub-G1	9.68	17.3	19
G0/G1	73.8	67.5	53.4
S	6.15	3.74	8.31
G2	10.3	11.7	19

	Cntr	TGF-β2	EMD
sub-G1	7.69	5.77	3.75
G0/G1	56.9	59	62.7
S	16	12.6	13
G2	19.5	22.7	20.5

Figure 25. Effect of TGF-β treatment and c-MET inhibition on viability and cell cycle progression. ZH-161, ZH-305 and T-269 were exposed to TGF-β2 (2 ng/ml) or EMD1214063 (1 µM) for 72 h. DMSO diluted in NB medium (1:20'000) served as a control. (A) Induction of cell death was evaluated by annexin V (AnnV)/PI staining. (B) Cell cycle phase distribution profiles were obtained by PI staining using flow cytometry. Cell fractions are shown for non-treated *versus* treated cells in histograms as well as bar graphs.

Detection and significance of intratumoral TGF-β and HGF/c-MET heterogeneity in glioblastoma

The findings of an inhibitory effect on HGF/c-MET by TGF-β *in vitro* prompted us to validate an association between TGF-β and c-MET pathways *in vivo* using human glioblastoma specimens. The specificity of the antibodies and the optimal conditions for immunohistochemistry were determined as previously described using paraffin-embedded positive and negative control cell lines (Fig. 13 and 14). *TGF-β2* mRNA-expressing ZH-305 cells were immunopositive whereas *TGF-β2* mRNA-negative ZH-161 cells were also negative by immunocytochemistry. Similar studies were performed to test the anti-p-c-MET antibody in ZH-161 and T-269 cells by immunoblot and immunohistochemistry. We co-stained glioblastoma tissue samples from a large cohort of 66 different glioblastoma patient for TGF-β2 (red) and p-c-MET (green). p-c-MET was detected in glioblastoma cells as well as in the vasculature (Fig. 26A). p-c-MET immunoreactivity was observed mainly in the membrane and less in the cytoplasm. TGF-β2 was localized intracellularly as well as in the extracellular space, showing a diffuse staining pattern (Fig. 26B and C). Double staining of TGF-β2 and p-c-MET allowed the classification of the tumor specimens into four groups (Fig. 26D). In line with our *in vitro* findings of a negative control by TGF-β of c-MET activation, the two antigens were rarely present simultaneously in the same tumor specimen (n=13). Moreover, co-expression of TGF-β2 and p-c-MET in the same tumor cells was rarely detected (Fig. 26C, see arrowhead), showing that these two antigens exhibit almost mutually exclusive staining patterns among patients and within the tumor from the same patient. Accordingly, a negative correlation was detected between TGF-β2 and p-c-MET ($r=-0.29$, $p=0.02$).

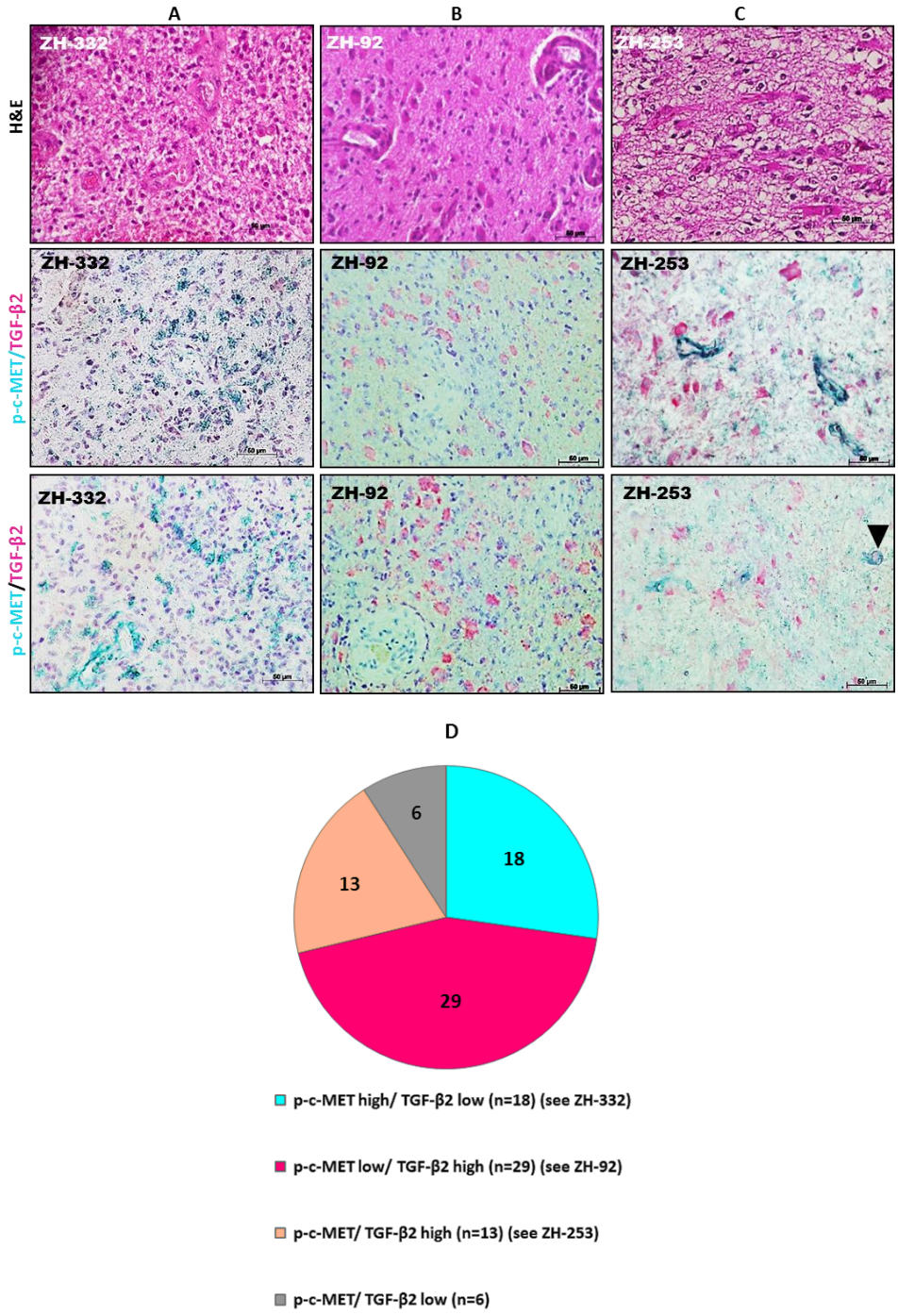


Figure 26. Simultaneous detection of TGF-β2 and phosphorylated c-MET in glioblastoma. Double immunostaining was performed on formalin-fixed, paraffin-embedded surgical glioblastoma specimens. (A-C) Representative images show hematoxylin and eosin (H&E) staining (upper panels); p-c-MET immunoreactivity in green (A and C, middle and lower panels) and TGF-β2 in red (B and C, middle and lower panels) in tumor regions of three patients. Nuclei are counter-stained with hematoxylin (blue). The scale bars correspond to 50 µm. (D) Graphical representation of the percentage of glioblastomas high for either TGF-β2 or p-c-MET or both.

Thus, we revealed a novel molecular and functional aspects of TGF-β - HGF/c-MET signal interaction shedding some light on a complex regulation of cancer cell self-renewal in glioblastoma (Fig. 27).

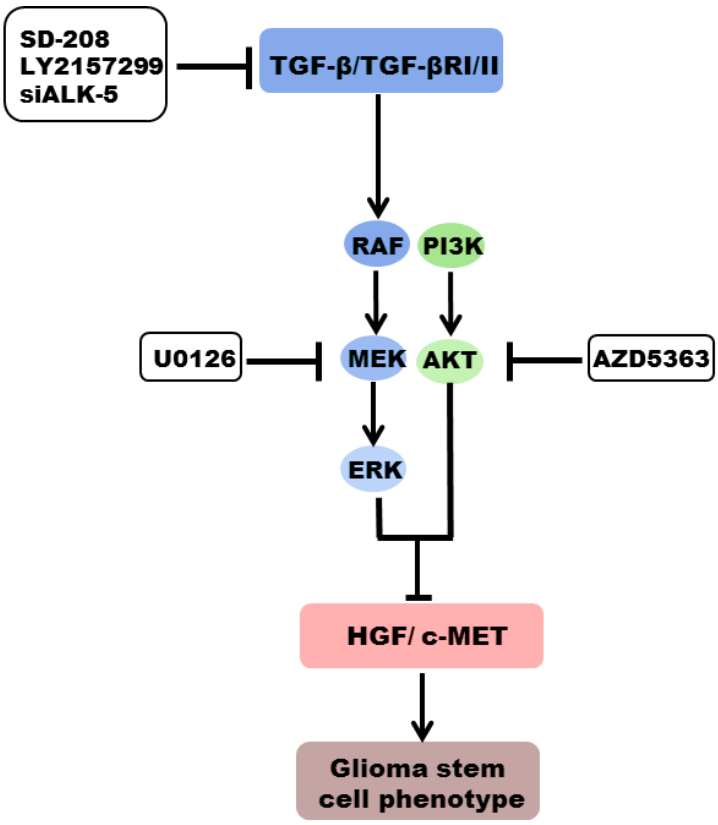


Figure 27. Overview of the crosstalk between HGF/c-MET and TGF-β pathways in glioblastoma. TGF-β inhibits c-MET activity through phosphorylation of MAPK/ERK kinase. The PI3K/AKT pathway is also involved in the regulatory mechanisms of c-MET activity by TGF-β in patient-derived GIC models. TGF-β inhibits stemness in GIC at least in part via its negative regulation of c-MET activity.

6.1.2 Discussion

Glioblastoma is a universally fatal type of cancer characterized by molecular diversity and intratumoral heterogeneity (Patel *et al.*, 2014). Potentially as a consequence of the functionally redundant cell signaling networks and multiple genetic alterations (Zheng *et al.*, 2012), glioblastoma remains largely refractory to current approaches of cancer therapy targeting specific signaling pathways (Prados *et al.*, 2015)**.** Given their role in mediating migration and invasiveness**,** resistance to irradiation and maintenance of the glioma stem cell pool, both HGF/c-MET and TGF-β signaling pathways are considered as potential therapeutic targets. Several approaches evaluated the inhibition of TGF-β signaling in glioma (www.clinicaltrials.gov) using antibodies, antisense oligonucleotides, ligand traps or TGF-β receptor kinase inhibitors (Han *et al.*, 2015). Similarly, many clinical trials explored a therapeutic role for HGF/c-MET inhibition. None of these approaches have been successful in the clinic (Awad *et al.*, 2014; Cloughesy *et al.*, 2017). Association between the TGF-β and HGF/c-MET signaling pathways has been demonstrated in other cancer types. The TGF-β pathway in fibroblasts suppresses mammary tumorigenesis by antagonizing HGF/c-MET signaling to promote scattering and invasion (Cheng *et al.*, 2007; Cheng *et al.*, 2008). Moreover, TGF-β negatively regulates *c-MET* and *HGF* mRNA levels by SMAD2-mediated transcriptional repression in human squamous carcinoma cells (Hoot *et al.*, 2013). In astrocytoma cells, TGF-β family ligands decrease HGF synthesis and secretion (Chattopadhyay *et al.*, 2004). However, the functional interactions between TGF-β family members and HGF/c-MET signaling in glioblastoma pathogenesis remained uncharacterized.

Here we have elucidated the crosstalk between the TGF-β and HGF/c-MET dependent signaling pathways in gliobastoma using three patient-derived GIC models as well as human glioblastoma specimens. We selected two models with constitutive c-MET activation, ZH-161 and ZH-305, and included a c-MET-negative model, T-269, as a reference (Fig. 12). We found that TGF-β stimulation reduced c-MET phosphorylation in ZH-161 and ZH-305 cells (Fig. 14). This was explained by a reduction of *HGF* mRNA expression and protein release (Fig 16). Exogenous HGF rescued c-MET pathway activity in the presence of TGF-β (Fig. 18). Additionally, repression of p-c-MET was associated with minor reduction of total c-MET in ZH-

305 cells. The effect of TGF-β on HGF/c-MET pathway activation was prevented upon pharmacological or genetic inhibition of ALK-5 (Fig. 14-15). Silencing of *SMAD2/3/4* gene expression did not interfere with the TGF-β-dependent control of the c-MET pathway. Conversely, both the blockade of the MAPK/ERK cascade by U0126 and of the PI3K/AKT signaling by AZD5363 attenuated the effect of TGF-β on c-MET phosphorylation as well as *HGF* gene expression (Fig. 20 and 21), suggesting that TGF-β activity on HGF/c-MET is regulated by both pathways. TGF-β directly phosphorylated ERK 1/2 (Fig. 20), thus defining this non-canonical signaling as the major mediator of the crosstalk between TGF-β and c-MET. In response to exogenous TGF-β, there was only minor or no stimulation of p-AKT (Fig. 21), indicating a lack of direct TGF-β-PI3K/AKT interaction in these GIC lines. Interestingly, induction of c-MET phosphorylation was observed upon the blockade of PI3K/AKT signaling or - to a lesser extent - upon MAPK/ERK inhibition alone, confirming a negative control of c-MET activity by AKT and MAPK/ERK, warranting clinical investigation to determine the significance of p-c-MET as an escape mechanism to AKT or MAPK/ERK inhibitors (Fig. 20 and 21).

The PI3K/AKT and ERK signaling cascades are activated in response to c-MET and are common downstream effectors of many receptor tyrosine kinases (Paumelle *et al.*, 2002; Organ and Tsao, 2011). Some evidence supports that PI3K/AKT and ERK act downstream of TGF-β, too (Zhang, 2009; Akhurst and Hata, 2012).

The low TGF-β pathway activity in these GIC models is reflected by undetectable phospho-SMAD2/SMAD3 by immunoblotting, furthermore by the unaltereted gene expression of the major effector and downstream target of TGF-β, *PAI-1*, upon *ALK-5* or *SMAD2/3/4* gene silencing (Fig. 15 and 19). Thus, reduced TGF-β can shift the balance in favor of c-MET receptor activation in these GIC lines. Notably, *PAI-1* transcriptional regulation is known to be stimulated by a variety of cytokines, including EGF (Kasza *et al.*, 2001; Paugh *et al.*, 2008) independenly of TGF-β, which can explain the relative high basal *PAI-1* mRNA levels in these cells.

In addition, the TGF-β and c-MET signaling pathways have been associated with the induction of stem marker factors essential for GIC maintenance (Ikushima *et al.*, 2009; Penuelas *et al.*, 2009; Anido *et al.*, 2010; Li *et al.*, 2011; Rath *et al.*, 2013). OCT4, NANOG and SOX-2 contribute to the hallmark characteristics of stem and putative cancer stem cells by activation of target genes that encode pluripotency and self-renewal mechanisms (Wang *et al.*, 2013). We observed that exposure to

TGF-β downregulated stem cell markers gene expression in c-MET-positive ZH-161 and ZH-305, but not in c-MET-negative T-269 cells (Fig. 22). This was paralleled by an according suppression of spherogenicity and minor changes in viability and cell cycle progression (Fig. 23 and 25). c-MET inhibition had the same effects as TGF-β stimulation in these assays. Conversely, T-269 did not respond to any manipulation of the TGF-β or c-MET pathways, showing a paradigm of how stem cell behavior is differentially regulated in different cellular contexts. As previously described (Moriyama *et al.*, 1996; Lamszus *et al.*, 1999; Walter *et al.*, 2002; Li *et al.*, 2011), we induced sphere formation by stimulation of c-MET-positive GIC models with HGF only in the absence of EGF and FGF supplementation. Additionally, under these culture conditions, the stimulation with HGF overcame the reduction in sphere formation observed upon exposure to TGF-β2 in ZH-161 and ZH-305 (Fig. 24). These findings may suggest that TGF-β suppresses stem cell characteristics by preventing c-MET overactivation in molecular sub-types of gliomas (Fig. 27).

Taken together, our observations allow predicting that human glioblastomas would tend to up-regulate either of both pathways but not both signaling simultaneously. This assumption was confirmed by a negative correlation between TGF-β2 and p-c-MET immunoreactivity upon double immunostaining in a large cohort of glioblastoma patients (Fig. 26). In fact, the modulation of c-MET pathway activity by TGF-β allows to speculate that subsets of patients with glioblastoma would have responded to TGF-β inhibitors such as galunisertib (LY2157299 monohydrate) with an increase of c-MET activity which might have counteracted any potential benefit from TGF-β pathway inhibition (Brandes *et al.*, 2016). Accordingly, c-MET negativity could be explored as a predictive biomarker for future clinical trials exploring TGF-β inhibition in glioblastoma.

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8. Curriculum vitae

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Education

Dates:	01/2014 - onwards
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Dates:	09/2010 - 12/2012
Title of qualification:	Master Degree in Biology Applied to Research in Biomedicine, University of Milan, Milan, Italy. Master thesis: “The absence of CCM induces Endothelial to mesenchymal transition via TGF-beta signaling pathway”. Supervisor: Prof. Elisabetta Dejana.

Dates:	09/2007 - 07/2010
Title of qualification:	Bachelor Degree in Biological Science, University of Milan, Milan, Italy. Bachelor thesis: “Investigation of the role of Senataxina on the progression of replication forks to TRX locus in S.cerevisiae. Supervisor: Prof. Marco Foiani.
Dates:	09/2002 - 07/2007
Title of qualification:	High School Scientific Diploma, Liceo Scientifico Statale F. Redi, Squinzano, Italy

Work experience

Dates:	01/2013 - 12/2013
Position:	Research associate fellowship supported by SEMM (European School of Molecular Medicine, http://www.semm.it)
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Fellowship

Research associate fellowship supported by SEMM (European School of Molecular Medicine, http://www.semm.it).

9. List of publication

Papa E, Weller M, Szabo E. Crosstalk of the HGF/c-MET and TGF-β pathways in glioblastoma. [Submitted].

Cuttano R, Rudini N, Bravi L, Corada M, Giampietro C, **Papa E**, Morini MF, Maddaluno L, Baeyens N, Adams RH, Jain MK, Owens GK, Schwartz M, Lampugnani MG, Dejana E. KLF4 is a key determinant in the development and progression of cerebral cavernous malformations. EMBO Mol Med. 2015; 10.15252.

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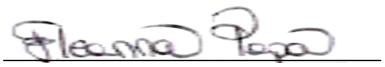
10. Declaration

I hereby declare that the content of this PhD thesis represents exclusively the product of my own work and has not been used for any other degree or diploma. All previously published materials from other have been specifically mentioned or inserted as citation. Any other contributions received have been acknowledged. Guidance in the project's realization and linguistic expressions are acknowledged to my thesis supervisors.

05.05.2017, Zürich



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